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Generating a link between a cell division and suicide gene provides a measure and solution for cell therapy safety

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The advent of human pluripotent cell lines holds enormous promise for the development of cell therapies to treat degenerative disease. Safety, however, is a crucial pre-condition for clinical application. Numerous groups have attempted to eliminate potentially harmful cells through the use of suicide genes¹; however, none of these efforts quantitatively define safety. Here, we show a concept for the protection of a suicide transgene system from inactivation and its realisation with genome engineering strategies. The strategy behind our fail-safe (FS) design is to create a transcriptional link between the suicide gene herpes simplex virus-thymidine kinase (HSV-TK) and a cell division essential gene, Cyclin-Dependent Kinase 1 (*CDK1*). Furthermore, we add a quantitative measure to cell therapy safety as the function of the cell number needed for a therapy and the type of genome editing performed. Even with the highly conservative estimates described here, we anticipate that our solution and the quantification of safety will rapidly accelerate the entry of cell-based medicine to the clinic.

Most randomly integrated transgenes are not stably expressed in all cells. The transfected cell population will show variegated expression as a result of epigenetic and genetic modifications, developmental regulation, mutations, and/or complete loss of the transgene²⁻⁴. To alleviate these mechanisms, we generated a transcriptional link between a cell division essential locus (CDEL) and a drug-inducible suicide system (SU), resulting in a CDEL-SU allele (Fig. 1a). In this design, the expression of the CDEL and SU are tightly linked together, and if required, the dividing cells of a graft can be eliminated or arrested through treatment with the SU prodrug. With an SU placed in a CDEL essential for cell-life, the entire graft could be eliminated regardless of its proliferative status.

According to the engineering definition, fail-safe systems are designed to mitigate potential harm caused by a system failure. Using this terminology, a cell population is considered fail-safe if all of the cells within the population contain a functional SU. The fail-safe level (FSL) is defined as a number. It indicates that the odds of using a non-FS batch of cells for therapy is one out of this number (Fig. 1b), such as one out of a thousand (FSL=1,000) or one out of a million (FSL=1,000,000) batches. Here, we used various *in vitro* and *in vivo* experiments as well as mathematical modelling to define the FSL that needs to be satisfied for future cell therapies to be designated as safe.

To generate a list of CDEL candidates, we cross-referenced genes whose knock-out has an early embryonic lethal phenotype in mice (<http://www.informatics.jax.org/phenotypes.shtml>) with data from a genome-wide CRISPR/Cas9 mutagenesis screen for essential genes in human cancer cell lines⁵. We found 167 genes with a high-fitness score (< -1.0)⁵ that also have a known early-embryonic lethal phenotype (Supplementary Table 1). For this study, we chose *CDK1* as our prototype CDEL and an optimised mutant version of HSV-TK, TK.007⁶ (TK from now on), as the SU prototype. Genetic studies have shown that *CDK1* is an essential kinase⁷. Its absence causes a block in the G2/M transition of the cell cycle, and other CDKs are not able to rescue its deficiency^{8,9}. TK has been extensively used for cell ablation¹⁰, and its mechanism of action in the presence of its clinically-approved prodrug ganciclovir (GCV), is well characterised¹¹. Importantly, *CDK1* and the linked TK are not expressed in non-dividing cells⁹, thus eliminating the potential immunogenicity of the viral TK protein¹² in quiescent donor cells.

To generate a transcriptional link between *CDK1* and TK (Fig. 1c), we used CRISPR/Cas9-assisted genome targeting to insert TK into the 3' untranslated region (3'UTR) of *CDK1* in mouse C2¹³ (Extended Data Fig. 1a-d, 2a-c), human H1¹⁴ (Extended Data Fig. 1e-i, 3a-f) and human CA1¹⁵ (Extended Data Fig. 4a-e) embryonic stem (ES) cells. Next, we determined the optimal GCV concentration for the heterozygous *CDK1*-TK mouse and human ES cells *in vitro* (Extended Data Fig. 2d, 3h), and then subsequently tested if we could control the growth of teratomas generated from these lines. Subcutaneous grafting of mouse ES cells into isogenic C57BL/6N recipients, and human ES cells into immunodeficient NOD scid gamma (NSG) recipients, resulted in teratoma formation with the expected efficiency (Extended Data Fig. 2e, 3g, 4d). When the volume of these tumours reached 500 mm³ (considered as day 0), we

administered GCV daily by intraperitoneal injection for up to four weeks. Exposure to GCV rendered the C2 ES cell-derived teratomas dormant, without any sign of growth rebound following the treatment (Fig. 1d, e and Extended Data Fig. 5a). The heterozygous H1 ES cell-derived teratomas in NSG mice responded similarly, albeit, in most of the cases, repeated and more extended GCV treatments were required to stabilise the teratoma size (Fig. 1f and Extended Data Fig. 5c). The observed decrease in teratoma size following GCV re-administration (Extended Data Fig. 5c) indicates that quiescent or slow-dividing cells within the teratoma began proliferating following drug withdrawal, and consequently, expressed TK and became sensitive to GCV. Furthermore, we found that the volume of the human teratomas frequently increased in the later phase of the experiments (Extended Data Fig. 5d); however, in agreement with previous reports^{16,17}, this increase was the result of extensive cyst formation (Extended Data Fig. 5d) and not solid tissue growth. The induced long-term dormancy of the teratomas was encouraging but also unexpected as we predicted that such a large ES cell-derived tissue ($\sim 10^9$ cells¹⁸) would contain hundreds of cells capable of escaping the SU through different types of mutations, such as silencing mechanisms and loss-of-heterozygosity (LOH). Within the well-encapsulated teratoma, however, it is likely that these assumed resistant cells (escapees) were eliminated by the known bystander killing effect of the TK/GCV system¹⁹.

To further investigate the capacity of the FS system to control cell proliferation, we also performed a breast cancer transplantation assay using heterozygous FS mammary epithelial tumour cells²⁰. Upon transplantation in an isogenic setting, we observed that after a delayed period (~ 100 days) the heterozygous FS tumours became resistant to GCV and continued growing in the presence of the drug (Extended Data Fig. 6a,b).

To identify escapees appearing in the expansion of heterozygous FS ES cells, we designed an *in vitro* experiment to mitigate the bystander killing effect (Extended Data Fig. 7a,b) and subsequently characterised the resistance mechanism in the eight independent clonal escapee-lines we obtained from 120 million cells. To determine if the escapee-generating mechanism was due to large genomic changes or *Cdk1*-TK locus-specific, we analysed the copy number (CN) of *Cdk1*, the TK transgene, and six additional endogenous genes all located on chromosome 10 (Fig. 1i, Extended Data Fig. 7d). Interestingly, this analysis revealed that only one escapee (E3 in Fig. 1i) contained the TK gene (Extended Data Fig. 7c). Further analyses did not detect mutations in the coding region of either *Cdk1* or TK (data not shown) but found the expression level of this allele was compromised and rendered the cells GCV resistant (Extended Data Fig. 7e). Another escapee, E5, was the result of a regional deletion involving the *Cdk1*-TK locus, and this led to at least an 18.5 Mbp hemizygous region in the wild type chromosome (predicted by CN=1 in *Sim1*, *Rhobtb1*, *Cdk1* and *Ank3* genes (Fig. 1i, Extended Data Fig. 7d). Regarding the other six clones, the diploidy (CN=2) of all of the examined endogenous chromosome 10 genes and the lack (CN=0) of TK suggested that these six escapees were formed by diploid LOH (dLOH). This was likely due to mitotic recombination events or chromosomal non-disjunction which homozygosed the wild type *Cdk1* allele in a diploid form. In summary, our data indicate that dLOH is the dominant mechanism of losing the *Cdk1*-TK allele in heterozygous ES cells. This finding is consistent with a study of mouse *Aprt* heterozygous cells, where dLOH accounted for 78% of the observed loss of gene function events²¹.

To mitigate the generation of escapees by dLOH, we established both mouse and human ES cells homozygous for the *CDKI*-TK allele (Extended Data Fig. 2a-c, 3a-h, 4a-e). As expected, we were unable to identify any escapees when we repeated the above experiment (Extended Data Fig. 7b). It was not surprising that the homozygous ES cell-derived teratomas behaved similarly to the heterozygous teratomas; a brief GCV treatment was sufficient to render these tissues dormant (Fig. 1g,h, Extended Data Fig. 5b,e). As observed for the human heterozygous lines, cyst formation also occurred in the human homozygous teratomas (Extended Data Fig. 5e). Furthermore, we also generated homozygous FS mammary tumour cells and, following transplantation, observed that the size of these tumors could be reduced and their growth restrained through GCV administration (Extended Data Fig. 6c). The observed

growth rebound following GCV withdrawal was not surprising as slow-dividing or quiescent, tumour-prone MMTV-PyMT cells survive GCV administration and can start growing in the absence of the drug. Nevertheless, even in this non-clinical, extreme situation where a tumour cell line is used for cell transplantation, the homozygous FS system is still capable of managing and controlling tumour growth. Since we were unable to identify any escapees appearing in homozygous FS ES cell expansion, we used Monte Carlo (MC) simulation to estimate the odds of generating an escapee in this scenario. The model considers three different types of mutations which could affect the function of the CDEL-SU link (Fig. 2a). Type 1 mutations (su1) render the SU non-functional while keeping the linked CDEL operational. Type 2 mutations (su2) eliminate both SU and CDEL functionality through epigenetic or genetic changes to the entire locus, including a hemizygous LOH-dependent mechanism. Type 3 mutations remove a functional CDEL-SU allele by dLOH.

To estimate the probabilities of the type 1, 2 and 3 mutations (p_1 , p_2 and p_3 , respectively) per cell generation (Fig. 2a), we used our targeted human H1 *CDK1*-TK-mCherry/*CDK1*-TK-EGFP dichromatic cell line, as most mutations in either *CDK1*-TK allele result in monochromatic cells. We grew 21 parallel cultures from a single dichromatic cell to an average of 5×10^6 cells per culture (> 22 consecutive doublings) and determined the number of monochromatic cells in the culture using flow cytometry. Next, we applied Luria-Delbruck fluctuation analysis^{22,23} to calculate the sum of the $p_1+p_2+p_3$ probabilities in the two *CDK1*-TK alleles. We found that the mutation rate of losing mCherry was 9.05×10^{-6} per cell per division while the mutation rate of losing EGFP was similar at 7.68×10^{-6} per cell per division (Extended Data Fig. 8a-b).

To further validate the probabilities of these various mutations, we also analysed published studies that focused on these events. In mouse ES cells, the mutation rate ($p_1+p_2+p_3$) of changing from a dichromatic to a monochromatic phenotype in the *Rosa26* locus (mouse Chr. 6) was 1×10^{-5} per cell, per division²⁴. Similarly, another study calculated the mutation rate of gene function loss in the *Gdf9* locus (mouse Chr. 11) to be 2.3×10^{-5} events per cell per division²⁵. Furthermore, the probability of the type 3 mutation, p_3 alone, has been calculated as 1×10^{-5} , 7.2×10^{-6} and 8.5×10^{-6} in three different studies²⁶⁻²⁸ by performing high G418 selection in mouse ES cells. The p_1+p_2 mutation rate has also been estimated in the human *HPRT* locus on the X chromosome to be $1.7-6 \times 10^{-7}$ by Luria-Delbruck fluctuation analysis²⁹, and 5×10^{-6} through mutation frequency analysis in population datasets³⁰.

Next, we performed MC simulation to establish the FSL of cell batches derived from different SU genotypes. Based on both published data and our own, we used the values $p_1=p_2=10^{-6}$ and $p_3=2 \times 10^{-5}$ per cell per division; all of which are intentional overestimates. Consequently, our calculated FSLs represent underestimates, being equal or lower than the actual FSL.

In silico, we subsequently generated a sufficient number of cell batches that were expanded from a single cell with an intact SU system. In every doubling, the model permits allele transitions (Fig. 2b) which determine the transition graph (depicted in Fig. 2c); reflecting the genotype change that could occur during cell expansion. For the homozygous *CDK1*-TK/*CDK1*-TK simulation, we initiated the batch production from an SU/SU cell, while for the heterozygous *CDK1*-TK/*CDK1*^{wt} simulation, the initiating cell was SU/su1. Lastly, for the compound heterozygous *CDK1*-TK/*CDK1*^{null} simulation, the initiating cell was SU/su2, since the su1 is functionally equivalent to *CDK1*^{wt}, while su2 is the same as a *CDK1*^{null} allele (Fig. 2a).

A batch of cells is considered FS if it does not contain any escapees (Fig. 2d). The MC simulation determined the frequency of getting a non-FS batch of cells, which allowed us to calculate the FSL as the function of the cell number needed for a therapeutic cell batch. Fig. 2e shows these functions for the different initiating cell genotypes detailed above.

The number of cells required for a cell therapy is disease-specific and is estimated to range between 10^5 (i.e. eye^{31,32}) and 10^{10} (i.e. heart³³) cells. The genotype scenarios presented in Fig. 2e shows that a single TK insertion (*CDK1*-TK/*CDK1*^{wt}, *CDK1*-TK/*CDK1*^{null}) gives a low FSL for the cell numbers

required for therapy. Contrarily, a homozygous TK insertion into *CDKI* significantly increases the FSL and brings the safety into a clinically relevant range for many future cell therapies. Diseases, such as arthritis, diabetes or myocardial infarction, however, require a larger number of therapeutic cells (10^8 to 10^{10} donor cells). In this range, even the homozygous CDEL-SU provided FSL is insufficient ($FSL < 10$). For this disease category, we propose the homozygous modification of two different CDELs with suicide genes. In this scenario, our MC simulation showed an enormous increase in FSL ($FSL > 10^6$ for all clinically relevant batch volumes, Fig. 2e).

We observed that the logarithm of FSL, as a function of the logarithm of the cell number, is a convex function. It is very close to a linear function when the FSL is above 10 and the cell number is in the clinically relevant range. Therefore, for an estimation of FSL, we applied linear regression to these segments (Fig. 2e). Interestingly, the slope of the two linear regressions (Fig. 2e) is very close to minus one (-0.99) and the y-intercepts of these lines were close to 9 and 16 for one and two CDEL modifications, respectively. Using these approximates, the calculation of FSLs becomes very simple, while remaining the desired underestimates (Fig. 2e):

For one CDEL: $FSL = 10^9/cn$, and for two CDELs: $FSL = 10^{16}/cn$, where cn is the cell number needed for a therapeutic cell batch.

Concerning the production of cells for a specific therapy, some are lost during differentiation or expansion. To account for this, the efficiency of cell production should be accurately estimated and the cell number needed to generate a therapeutic batch should be corrected accordingly.

In future autologous cell therapies, the generation of multiple therapeutic batches might not be necessary, and a single FS clone could be grown up to the number of cells required for therapy. On the other hand, if allogeneic cells are desired or HLA haplobanks³⁴ of human pluripotent cell lines will be operational, the generation of an off-the-shelf bank of cell batches would be advantageous. In this scenario, it would be more practical and economical to produce a large pool of cells which, following quality control (QC) analysis, could be aliquoted into therapeutic doses. Consequently, we calculated the effect of aliquoting on FSL using both mathematical (Supplementary Calculation 1) and Monte-Carlo modeling approaches. On the basis of our established *in silico* model (Fig. 2e), we simulated the process of escapee accumulation until the final pool size reached 2^{20} and 2^{27} cells. In both of these scenarios, we calculated escapee generation frequency distributions. Next, we randomly picked A aliquots from these pools and calculated the mean number of “bad” aliquots (containing one or more escapee) in each case. By dividing the overall number of aliquots A by the number of bad aliquots, we calculated the aliquot FSL (FSL_{ap}). The ratio between FSL_{ap} and the FSL of the cell population size $2^{20}/A$ or $2^{27}/A$ (FSL_a), is shown in Fig. 2f (red and orange lines, respectively). To confirm the results of this approach, we also calculated the same ratio using only theoretical assessment, where the final equation is as follows (Fig. 2f, blue line):

$$\frac{FSL_{ap}}{FSL_a} \approx \frac{1}{A(1 - \sum_{k=0}^{m-1} 2^{-k-1} (\frac{A-1}{A})^{2^k})}$$

where A is the number of aliquots generated from the pool of cells, and m is the integer part of the base 2 log of the aliquot size.

The decrease in FSL is largest when a small number of aliquots are used (Fig. 2f, blue line). Within a practical range of aliquots (100-500), the FSL of the batches remains almost constant, with the probability modeling slightly underestimating the FSL drop. Consequently, aliquoting results in an approximately five-fold decrease in FSL using both approaches. The difference between aliquoting from a 2^{20} ($\sim 10^6$) vs. 2^{27} ($\sim 10^9$) pool shows that the drop is moderately larger (Fig. 2f) when we generate aliquots from a pool size that is close to the edge of the linear part on the original FSL graph (Fig. 2e).

Following the generation of every cell batch or pool, a quality control (QC) needs to be performed to ensure that the originating cell was FS and consequently, the FSL calculation was correct. To

demonstrate the practicality of the QC process, we grew several batches of cells from a single, homozygous FS ES cell. At the early phase of expansion, we verified that both *CDKI*-TK alleles were expressed and intact in the batch-originating single cell using flow cytometry and through allele-specific PCR (Extended Data Fig. 9) and sequencing of the TK-coding region.

Both mouse and human ES cells with the homozygous modification of *CDKI* have normal ES cell morphology and self-renewing capacity in standard ES cell culture conditions (Fig. 3a,b). The cells differentiated into the three embryonic germ layers in teratomas (Fig. 3c,d). Furthermore, we generated chimeric mice from the *Cdk1*-TK heterozygous mouse ES cells, and after germline transmission, obtained viable heterozygous and then homozygous adult mice (Fig. 3e, Extended Data Fig. 6a). Homozygous FS H1 human ES cells express pluripotency markers (Fig. 3f), and were successfully differentiated into retinal pigmented epithelial (RPE) cells³⁵ (Fig. 3g-i), adipocytes, osteocytes, chondrocytes (Fig. 3j), definitive endoderm (Fig. 3k), pharyngeal pouch endoderm³⁶ (Fig. 3l) and beating cardiomyocytes (Supplementary Video 1). Additionally, using *in vitro* neural differentiation, we demonstrated the selective killing of dividing cells by the FS system. As expected, following a brief GCV treatment, all mitotically active cells were eliminated while non-dividing cells were spared (Fig. 3m). This ability could represent a valuable safety measure prior to the transplantation of cells into a recipient.

Although we previously demonstrated the robustness of the FS system using a breast cancer transplantation assay, we also wanted to simulate a clinical cell transplantation scenario gone awry. To accomplish this, we subretinally injected a 3:1 mixture of human homozygous FS RPE cells and human homozygous FS ES cells (mCherry tagged) into the eyes of NSG mice. Among 4 injected eyes, we did not observe any cell growth from the ES cell component of the graft when GCV was administered 24 hours post-injection for a period of 28 days, as a preventative measure (Fig. 4a-b, Extended Data Fig. 10a-c). In contrast, cell growth was detectable in 6 eyes that received an initial PBS treatment (Fig. 4c, Extended Data Fig. 10e-i). Importantly, even when GCV administration is delayed three weeks post-injection and cell growth has already been detected, the homozygous FS system efficiently stopped the growing ES cell-derived component of the graft; only non-dividing cells remained (Fig. 4c, Extended Data Fig. 10g-i). This experiment illustrates the ability of the FS system to selectively eliminate proliferating cells after cell transplantation. Furthermore, neither the initial nor the delayed GCV treatment affected the RPE graft or the integrity of the surrounding retinal tissue (Fig. 4b,c, Extended Data Fig. 10).

No therapy is without risk. Understanding and quantifying the risks associated with cell-based therapies is critical for the clinical advancement of regenerative medicine. Without a measurable safety system, it remains a challenging ambition to develop cell therapies that can treat degenerative diseases with an acceptable level of safety. We have developed a genome-editing strategy that allows a fundamental definition of safety, as well as a quantification of the safety level as a function of the cell number needed for any given cell therapy.

After transcriptionally linking a cell suicide element to a cell division essential locus, we mathematically defined and quantified the safety of our FS system. These calculated risks are highly conservative, and represent an underestimate of the actual safety level of the system, as they were performed using worst-case scenarios. This is necessary because the cells required for therapies (100 thousand to 10 billion) will need to be grown *ex vivo*. These conditions entail serious risks compared to most *in vivo* settings, where functional immune cells constantly surveil and eliminate transformed cells³⁷. Additionally, since the number of cells needed will necessitate a similar number of genome duplications, any possible mutation could arise. Some mutations, such as a dominant negative heterozygous P53 can lead to a growth advantage³⁸ that allows mutant cells to rapidly take over the entire population within a competitive culture environment. For these reasons, the safety of *in vitro*-generated therapeutic cells must be vigorously assessed.

The concept and genome-editing approach presented here not only addresses these concerns, but also allows one to define cell therapy safety in a quantitative way. We contend that the risks, as determined

through experimentation and calculation, are sufficiently low and that the FS system will become an indispensable component of prospective cell therapies. Moreover, our approach to assess and quantify the safety of cell-based therapies will be critical for informed decision making by the regulators, clinicians and patients who will advance the therapies that will transform modern medicine.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions Q.L. and C.M. designed and conducted most of the experiments, analysed the data, and wrote the manuscript. M.V.S. conducted the Monte Carlo simulations and analysed the data. I.G. performed the mathematical modelling. E.J.N. conducted experiments and wrote the manuscript. S.H. performed the eye experiment and analysed the data. H.Y., C.K., P.Z., C.L., K.N., M.M., H.S. conducted experiments. A.N. conceived and supervised the study, designed experiments, analysed the data and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. A.N., C.M. and Q.L. are inventors on a patent application covering the FS technology (PCT/CA2016/050256). A.N. is the co-founder and shareholder of panCELLa Inc. C.M. is a senior scientist at panCELLa Inc. The other authors declare no competing interests. Correspondence and requests for materials should be addressed to A.N. (nagy@lunenfeld.ca).

Methods

Generation of targeting vectors. Targeting vectors were generated by DNA synthesis, molecular cloning, recombineering and the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs).

Generation of CRISPR/Cas9 vectors. pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230)³⁹. Guide sequences for CRISPR/Cas9 were analyzed using the online CRISPR design tool (<http://crispr.mit.edu>). Guide sequence for mouse *Cdk1* targeting: TAAGAAGATGTAGCCCTC. Guide sequence for human *CDK1* targeting: CTATCTGTTGACATAACATA.

Mouse ES cell culture. C57BL/6N C2 ES cells were grown at 37°C in 95% air 5% CO₂ on MEFs obtained from TgN(DR4)1Jae/J mice (<http://jaxmice.jax.org/strain/003208.html>) at all times except for one passage on gelatinized tissue culture plates prior to aggregation⁴⁰. Two types of media were used: 1). FBS-DMEM ES cell medium for gene targeting consisted of high glucose DMEM supplemented with 15% FBS (previously shown to support germline chimera generation), 2 mM GlutaMAX, 1 mM Na Pyruvate, 0.1 mM non-essential amino acids (NEAA), 50 U/ml penicillin and streptomycin (all Thermo Fisher Scientific), 0.1 mM 2-mercaptoethanol (Sigma) and 1000 U/ml LIF prepared with LIF producing plasmid.⁴¹ 2). KOSR+2i medium was used for 2-4 passages prior to the generation of ES cell chimeras¹³. KOSR+2a consisted of high glucose DMEM medium supplemented with 15%

KnockOut Serum Replacement (KOSR) (Thermo Fisher Scientific), 1 mM Na Pyruvate, 0.1 mM NEAA, 0.1 mM 2-mercaptoethanol, 2 mM GlutaMAX, 50 U/ml penicillin/streptomycin, 500 U/ml LIF, 5 mg/ml Insulin (Thermo Fisher Scientific), 1 μ M of the mitogen-activated protein kinase inhibitor PD0325901 (StemGent) and 3 μ M of the glycogen synthase kinase-3 inhibitor CHIR99021 (StemGent). Cells were fed daily and passaged when they reached a confluency of 70-80%. 0.05% Trypsin-EDTA (Thermo Fisher Scientific) was used for the passage of cells grown in FBS-DMEM and Accutase (STEMCELL Technologies) was used for cells grown in KOSR+2i medium.

Mouse ES cell targeting. 50,000 mouse C57BL/6N C2 ES cells were transfected with 2 μ g DNA (Mouse Target Vector 1 or 2: 1.5 μ g, CRISPR vector: 0.5 μ g) by JetPrime transfection (Polyplus). The cells were selected for G418-resistance (160 μ g/ml) starting 48h after transfection. Resistant clones were picked independently and replicated in 96-well plates for freezing and genotyping with PCRs. PCR-positive clones were expanded, frozen to multiple vials, and genotyped by Southern blotting.

Selection cassette excision in mouse ES cells. Correctly targeted ES cell clones were transfected with episomal-hyPBase (for Mouse Target Vector I) or pCAGGs-NLS-Cre-IRES-puromycin (for Mouse Target Vector II). 2-3 days following transfection, cells were trypsinized and plated clonally (1,000 - 2,000 cells per 10 cm plate). mCherry-positive clones were picked and transferred to 96-well plates independently and genotyped by PCR and Southern blotting to confirm the excision event. The junctions of the removal region were PCR-amplified, sequenced, and confirmed to be intact and without any frameshift mutations. GCV (Sigma) to test for TK activity was used at a final concentration of 1 μ M.

Human ES cell culture. Human CA1 and H1 ES cells were cultured on Geltrex (Thermo Fisher Scientific) using mTeSR1 media (STEMCELL Technologies) containing 50 U/ml penicillin/streptomycin (Thermo Fisher Scientific). Cells were passaged using Tryple Express (Thermo Fisher Scientific) and were subsequently plated in mTeSR media containing 10 μ M ROCK inhibitor (Selleckchem) for 24 hours.

Human ES cell targeting. For Human Targeting Vectors I & II, 6 million human ES cells were electroporated using Neon Transfection System (Thermo Fisher Scientific) with protocol 14 (pulse voltage: 1200 mV, pulse width: 20 ms, pulse number: 2) with 24 μ g DNA (Target Vector: 18 μ g, CRISPR vector: 6 μ g). After transfection, cells were plated on four 10 cm plates. G418 selection at 30 μ g/ml or puromycin selection at 0.75 μ g/ml was initiated 48h after transfection. Independent colonies were picked to 96-well plates and each plate was duplicated for further growth and genotyping with PCR. PCR-positive clones were expanded, frozen to multiple vials, and genotyped with Southern blotting. For Human Target Vector III targeting, 10 million human ES cells were electroporated using Neon protocol 14 with 40 μ g DNA (Human Target Vector III: 30 μ g, CRISPR vector: 10 μ g), and plated in four 10 cm plates. 3-4 days after transfection, cells that were double-positive for mCherry and eGFP were sorted into one well of a 96-well plate. After recovery from FACS, cells were dissociated and plated clonally (1,000 - 2,000 cells per 10 cm plate). Next, clones were picked independently, replicated, and transferred to 96-well plates for freezing and genotyping with PCR. PCR-positive clones were expanded, frozen to multiple vials, and genotyped by Southern blotting.

Selection cassette excision in human ES cells. 1 million correctly targeted ES cell clones were electroporated with 2 μ g episomal-hyPBase-IRES-puro (for Human Target Vector I) or 2 μ g episomal-Cre-IRES-puro (for Human Target Vector II) using Neon protocol 14. Once the cells were confluent in 6-well plates, mCherry-positive cells were sorted to one well of a 96-well plate by FACS. After recovery, cells were dissociated and plated clonally (1,000 - 2,000 cells per 10 cm plate). Clones were

471 picked and transferred to 96-well plates independently and genotyped by PCR and Southern blotting
472 to confirm the excision event. The junctions of the removal region were PCR-amplified, sequenced
473 and confirmed to be intact and without frameshift mutations.

474 **PCR genotyping.** 2X Taq PCR master mix (Biomart) was used for all PCR reactions. Genomic DNA
475 from human cell pellets was extracted using the DNeasy Blood & Tissue Kit (Qiagen). The primer
476 pairs and conditions used for each reaction are listed in Supplementary Table 2.

477 **Southern blotting.** 10 µg of genomic DNA was extracted from PCR-positive clones, digested with
478 ScaI-HF overnight, resolved by 0.6 - 0.7% gel electrophoresis, and transferred to Hybond N+ (GE
479 Healthcare). The following probes were labelled with ³²P and used to hybridize with the membrane
480 (~25 ng probe per ml hybridization solution). Human *CDK1* genomic probe: PCR amplified with
481 primers h*CDK1*-Probe6-F + h*CDK1*-Probe6-R. Mouse *Cdk1* genomic probe: PCR amplified with
482 primers 647302FWD + 647302REV. mCherry probe: the entire length of mCherry. eGFP probe: the
483 entire length of eGFP. TK-mCherry probe: cut from h*CDK1*-PB-neo-TK-mCherry with
484 Bsu36I+SgrAI: 1092 bp, gel-purified.

485 **Mice.** The CD-1 (ICR) (Charles River) outbred albino stock was used as embryo donors for aggregation
486 with ES cells and as pseudopregnant recipients. C57BL/6NCrl (Charles River) was used as the host for
487 teratoma assays with mouse C2 ES cells. C57BL/6NCrl or B6N-Tyr_c N4/Crl#493 (Charles River)
488 was used as the host for mammary fat pad transplantation of mammary epithelial cells. NOD scid
489 gamma /J#5557 (Jackson Laboratories) was used as the host for teratoma assays with human H1 or
490 CA1 ES cells. FVB/N-Tg(MMTV-PyVT)634Mul/J was a gift from Dr. William Muller's lab²⁰, and the
491 backcross to B6J background was done by Dr. Anthony Pawson's lab. Animals were maintained on a
492 12 h light/dark cycle and provided with food and water *ad libitum* in individually ventilated units
493 (Techniplast) in the specific-pathogen free facility at The Centre for Phenogenomics (TCP). All
494 procedures involving animals were performed in compliance with the Animals for Research Act of
495 Ontario and the Guidelines of the Canadian Council on Animal Care.

496 **Generation of chimeras and mouse lines.** Morula aggregations were performed as previously
497 described⁴⁰. Chimeras were identified at birth by the presence of black eyes and later by coat
498 pigmentation. Male chimeras with more than 50% ES cell contribution to coat colour were bred with
499 CD-1 females to identify germline transmitter. The transmitter was then bred with C57BL/6NCrl
500 females and pups were confirmed by genotyping to obtain *Cdk1*-TK/*Cdk1* mice. *Cdk1*-TK/*Cdk1*
501 MMTV-PyMT males were generated by breeding MMTV-PyMT (B6) males and *Cdk1*-TK/*Cdk1*
502 females. *Cdk1*-TK/*Cdk1*; MMTV-PyMT and *Cdk1*-TK/*Cdk1*-TK; MMTV-PyMT female mice were
503 generated by breeding *Cdk1*-TK/*Cdk1*; MMTV-PyMT males and *Cdk1*-TK/*Cdk1* females.

504 **Teratoma assay.** Matrigel Matrix High Concentration (Corning) was diluted 1:3 with cold DMEM
505 media on ice. 1-5 million mouse ES cells or 5-10 million human ES cells were suspended in 100 µl of
506 Matrigel-DMEM and injected subcutaneously into one or both dorsal flanks of C57BL/6NCrl mice
507 (for mouse C2 ES cells) and NOD scid gamma /J#5557 mice (for human H1 and CA1 ES cells).
508 Teratomas formed 2-4 weeks after injection. Teratoma size was measured using calipers and volume
509 was calculated using the formula $V = (L \times W \times H) \pi / 6$. GCV /PBS treatment was performed through daily
510 intraperitoneal injections (50 mg/kg) with varying treatment durations. At the end of treatment, mice
511 were sacrificed and tumors were dissected and fixed in 4% paraformaldehyde for histological analysis.

512 **Breast cancer transplantation assay.** *Cdk1*-TK/*Cdk1*; MMTV-PyMT and *Cdk1*-TK/*Cdk1*-TK;
513 MMTV-PyMT female mice developed mammary gland tumours between 3-6 months old. Mammary
514 epithelial tumorigenic cells were isolated from developed tumours through digestion in 10×
515 collagenase/hyaluronidase (STEMCELL Technologies), and dilution to 1x with media consisting of
516 DMEM/F12 (Thermo Fisher Scientific) + 10% FBS + 50 U/ml penicillin/streptomycin for an hour in
517 37 degree. The digested cells were washed and pelleted with DMEM/F12 + 10% FBS 4 times, and
518 plated in CnT-PRIME epithelium culture media (CELLnTEC Advanced Cell Systems AG) on plates
519 coated with 0.1% gelatin (Sigma). Without passaging, primary mammary epithelial cells were
520 dissociated and re-suspended in PBS at 10,000 cells per µl, and 50 µl (500,000 cells) was transplanted
521 to each mouse by intraductal injection after making a small abdominal skin incision as previously
522 described⁴². Tumour measurement and PBS/GCV treatment were the same as described in the teratoma
523 assay.

524 **Differentiation of human ES cells into retinal pigmented epithelial (RPE) cells.** RPE differentiation
525 was performed as previously described³⁵ with minor changes. Human ES cells were plated on Geltrex-
526 coated 6-well plates and cultured in feeder-free conditions with mTeSR medium until confluency was
527 reached and the colonies lost their tight borders (7-10 days). Next, the media was replaced with
528 differentiation media (basal media with 13% KOSR) and changed every 2-3 days. The basal media
529 consisted of KO-DMEM supplemented with 50 U/ml penicillin/streptomycin mix, 1 mM Na Pyruvate,
530 0.1 mM NEAA, 2 mM of GlutaMAX and 0.1 mM 2-mercaptoethanol. Initial pigmentation was
531 observed approximately 3 weeks after the switch to differentiation media. Clusters of RPE cells were
532 manually picked and transferred to a Geltrex-coated 24-well plate (3 clusters/well) when they were
533 large enough (~1mm in diameter) for enrichment and the media was changed to RPE media, consisting
534 of basal media with 5% FBS, 7% KSR and 10 ng/ml bFGF (Peprotech).

535 **Differentiation of human ES cells into definitive endoderm.** Definitive endoderm differentiation
536 was performed using the STEMdiff Trilineage Differentiation Kit (StemCell Technologies), and
537 characterized by immunostaining for SOX17 and FOXA2 (Supplementary Table 3).

538 **Differentiation of human ES cells into pharyngeal pouch endoderm (PPE).** PPE differentiation
539 was performed as previously described³⁶ with the only modification being that the induction from ES
540 cells to definitive endoderm is one day shorter than reported.

541 **Differentiation of human ES cells into mesenchymal stem cells (MSCs) and subsequent**
542 **adipogenic, osteogenic and chondrogenic differentiation.** ES cells were cultured in mTeSR medium
543 for 2 days. Next, cells were induced into early mesoderm progenitor cells with STEMdiffMesenchymal
544 Induction Medium (STEMCELL Technologies) for 4 days and then were maintained with MesenCult-
545 ACF Medium (STEMCELL Technologies). Cells were continually passaged onto 6-well plates pre-
546 coated with MesenCult-ACF Attachment Substrate (STEMCELL Technologies) to derive early
547 mesenchymal progenitor cells. At day 21, the MSCs showed a fibroblast-like morphology and the
548 culture medium was changed every 3 days. For adipogenic differentiation, MSCs at a density of 20,000
549 cells/well were plated with MesenCult-ACF Attachment Substrate and cultured with MesenCult-ACF
550 Medium for 2 days. Adipogenesis was induced using the StemPro Adipogenesis Differentiation Kit
551 (Thermo Fisher Scientific). After 21 days, lipid droplets were visualized using Oil Red O (Sigma). For
552 osteogenic differentiation, ES-derived MSCs at the density of 50,000 cells/well were plated with
553 MesenCult-ACF Attachment Substrate and cultured with MesenCult-ACF Medium for 2 days.
554 Osteogenesis was induced using the StemPro Osteogenesis Differentiation Kit (Thermo Fisher
555 Scientific). After 21 days, calcium deposition was visualized using Alizarin Red (Sigma). For induction
556 of chondrogenic differentiation, ES-derived MSCs were centrifuged in 15 ml conical tubes at 500g for

557 5 mins to create cell pellets with 5,000,000 cells per pellet. Chondrogenesis was induced using the
558 StemPro Chondrogenesis Differentiation Kit (Thermo Fisher Scientific). After 21 days, cartilage was
559 visualized using Alcian Blue (Sigma). Differentiation media was changed every 3 days.

560 **Differentiation of human ES cells into beating cardiomyocytes.** Cardiomyocyte differentiation was
561 performed using STEMdiff Cardiomyocyte Differentiation Kit (STEMCELL Technologies).

562 **Differentiation of human ES cells into neuronal progenitors and neurons.** To differentiate human
563 ES cells into neuronal progenitors, human ES cells were plated at 50-100,000 cells per cm² in 1:1
564 DMEM/F12 : Neurobasal (Thermo Fisher Scientific), 0.5x N2 supplement (home-made, 1.92 mg/ml
565 putrescine, 2.376 µg/mL progesterone, 3.6 µM Selenium, 10 mg/mL Apo-transferrin, 0.75% BSA, 20
566 g/ml insulin), 0.5x B27 supplement with Vitamin A (Thermo Fisher Scientific), 2 mM Glutamax, 0.1
567 mM Beta-mercaptoethanol, 50 U/ml penicillin/streptomycin, 10 µM SB431542 (Selleckchem), 100
568 nM LDN193189 (Selleckchem) (+10 µM ROCK inhibitor overnight only, then removed). Cells were
569 maintained in this condition for 8 days and media was changed every other day. Next, neuronal
570 progenitors were dissociated with Accutase and plated at a density of 5x10⁴ cells per cm² on laminin
571 (Sigma, 1 µl for 1cm², diluted in 250 µl PBS without Ca and Mg) in fast neuron differentiation media,
572 1:1 DMEM-F12: Neurobasal, 1x B27 supplement with Vitamin A, 5 µM DAPT (Selleckchem), 2 mM
573 Glutamax, 0.1 mM Beta-mercaptoethanol, 50 U/ml penicillin/streptomycin. Media were changed every
574 three days. 10 µM GCV was added 6 days after neuron differentiation and kept for 6 days. 10 µM BrdU
575 (Sigma) was added after 5 days of GCV treatment and the cultures were fixed after 6 days of GCV
576 treatment and were immunostained for BrdU and beta-TubulinIII (Supplementary Table 3).

577 **Flow cytometry analysis and fluorescence-activated cell sorting (FACS).** Flow cytometry and
578 FACS were both performed and analyzed by the Lunenfeld-Tanenbaum Research Institute flow
579 cytometry facility. FACS was performed using the ASTRIOS EQ cell sorter. Flow cytometry was
580 performed using the GALLIOS flow cytometer and evaluated using Kaluza Analysis Software
581 (Beckman Coulter). Samples were gated for live single cells using forward scatter, side scatter and
582 DAPI staining. Wild-type and single-color samples of the same cell type as the experimental samples
583 were used for negative controls and compensation calculations. Human ES cell samples were single-
584 cell sorted using StemFlex Medium (Thermo Fisher Scientific) plus 10 µM ROCK inhibitor.

585 **Immunostaining.** 4% PFA-fixed cells were blocked and permeabilized with 5% goat serum + 1M
586 Glycine + 1% Triton X-100 (all Sigma) in PBS without Ca and Mg or animal-free blocker (Vector
587 Laboratories) + 1% Triton X-100 in milliQ water. All of the primary antibody information can be found
588 in Supplementary Table 3. Staining was visualized using a Zeiss LSM780 confocal microscope.

589 **Histology analysis.** Paraffin embedding, paraffin block sectioning, and H&E staining were performed
590 by the Pathology Core of The Centre for Phenogenomics.

591 **Quantitative PCR.** Gene expression analyses were completed as follows: RNA extraction by
592 GenElute™ Mammalian Total RNA Miniprep Kit (Sigma), reverse transcription with QuantiTect
593 Reverse Transcription Kit (Qiagen), Q-PCR with SensiFAST SYBR No-Rox Kit (Bioline) on Bio-Rad
594 CFX Real-Time Systems (Bio-Rad), and analysis with Bio-Rad CFX Manager 3.1. All the information
595 of primers and probes for the TaqMan Q-PCRs can be found in Supplementary Table 2. The reactions
596 were performed using TaqMan Genotyping Master Mix (Thermo Fisher Scientific) and CFX Real-
597 Time Systems, and were analyzed by CopyCaller Software v2.1 (Thermo Fisher Scientific).

598 **Luria and Delbruck assay.** The Luria and Delbruck assay was performed as previously described²².
599 *CDK1*-TK homozygous 3C cells were single-cell plated in a 96-well plate using FACS. 21 single cell-
600 derived cultures were grown to an average of 5 million cells per culture, and the number of single-
601 positive cells in each culture were analyzed using flow cytometry. The mutation rate was calculated
602 using the previously described equation²³ in <https://www.wolframalpha.com/>.

603 ***In vivo* transplantation of RPEs.** *CDK1*-TK homozygous 3C ES cells were transfected with PB-
604 CAGGs-mCherry-pA plasmid and sorted for high expressors. 40,000 3C-derived RPEs only or 30,000
605 3C-RPEs plus 10,000 mCherry-tagged 3C ES cells were injected subretinally with 0.5%/0.5% (wt/vol)
606 hydrogel blend of hyaluronan and methylcellulose (HAMC) in HBSS. PBS or GCV (50 mg/kg)
607 treatment were started the day after cell injection and were given every other day through
608 intraperitoneal injections. Monitoring by funduscopy and optical coherence tomography were
609 performed on the day after transplantation and then once a week.

610 **Monte Carlo simulation.** An ES cell population was considered as a mix of mutant and non-mutant
611 cells with reference to the CDEL-SU locus (or loci). All possible mutations were categorized into three
612 different types: type 1, when only the SU part of the locus becomes non-functional (*su1* allele); type 2,
613 when both the CDEL and SU become non-functional (*su2* allele); type 3, when any of the above occurs
614 as a result of LOH (Fig. 2a,b). Back mutations, such as *su1*→SU or *su2*→SU or *su2*→*su1*, were
615 not considered, due to their extremely low probabilities (Fig. 2c). Back mutations, like SU/*su1* →
616 SU/SU, SU/*su2* → SU/SU and *su1*/*su2* → *su1*/*su1*, were considered as a part of the more frequent LOH
617 process. *p1*, *p2*, and *p3* were designated the probabilities of each mutation type, respectively. We
618 distinguished between two types of *p3*: *p3mr* (probability LOH occurred through mitotic
619 recombination), where both daughter cells survive; and *p3cnd* (probability LOH occurred through
620 chromosomal non-disjunction), where one of the daughter cells with the single remaining copy of the
621 chromosome is likely to die⁴³. On the basis of these probabilities, matrices of transitions between all
622 possible genotypes within one or two CDEL systems were constructed (Fig. 2c,
623 <https://github.com/mashutova/failsafe>).

624 With each division cycle (*d*), all cells within the population except cells with the *su2*/*su2*
625 genotype, were allowed to divide. Genotypes of *su1*/*su2* and *su1*/*su1* were considered escapees and the
626 simulation initiated from one non-mutant cell. *N*(*g1*,*d*) was the number of cells of genotype *g1* at
627 doubling *d*, and *p*(*g1*,*g2*) was the probability of transition from genotype *g1* to *g2*. In each doubling the
628 number of cells changing genotype from *g1* to *g2* was determined through random sampling from a
629 binomial distribution with parameters $2 \cdot N(g1, d-1)$ and *p*(*g1*,*g2*). We used Poisson approximation of
630 binomial distribution to work with ultra-low *p*(*g1*,*g2*). For each division, the number of cells of each
631 genotype was assessed, and the simulation proceeded until the first escapee was detected.

632 For each starting genotype, we performed more than 10 million simulations and obtained a
633 distribution of the number of doublings from the detection of the first escapee. On the basis of this data,
634 we generated a function of FSL (overall number of trials divided by number of trials with escapees)
635 over cell population size (2^d) (Fig. 2d). Since all graphs contain almost linear regions, we used linear
636 models to extrapolate them to high FSL values. To obtain linear regression lines, we only used
637 simulated points from the linear-like part of the graph (*R*-squared > 0.999) with 95% confidence
638 intervals less than 1000. To obtain a conservative boundary for the FSL, we used only the lowest CI
639 values to build linear regressions.

To analyse the outcome from the aliquoting of the pool of fail-safe cells possibly containing escapees, using probability modelling we developed the following formula to calculate the drop of FSL (for details see Supplementary Calculation 1)

$$\frac{FSL_{ap}}{FSL_a} \approx \frac{1}{A(1 - \sum_{k=0}^{m-1} 2^{-k-1} (\frac{A-1}{A})^{2^k})}$$

To reduce the complexity of the model, we considered only one escapee event in the pool, as the possibility of two independent escapees occurring in a pool is low in the quasi linear phase of FSL. Nevertheless, we tested the effect of this omission on the drop of FSL due to aliquoting using Monte Carlo simulation. We performed 10 million independent trials for a doubling of 20 and a doubling of 27, and obtained a distribution of the number of escapees for each of them. Through randomly sampling a number of escapees from each trial to the A aliquots, we calculated the number of “bad” aliquots containing one or more escapees (Ab). To calculate a new FSL of the population after aliquoting, where FSLp is the FSL of the original population, we used the formula $A * FSL_p / \text{mean}(Ab)$. The drop in FSL was measured *in silico* and was compared with the one we obtained from the equation obtained from the probability model.

Data availability. The DNA sequences of the vectors and plasmids used in this study are available upon request.

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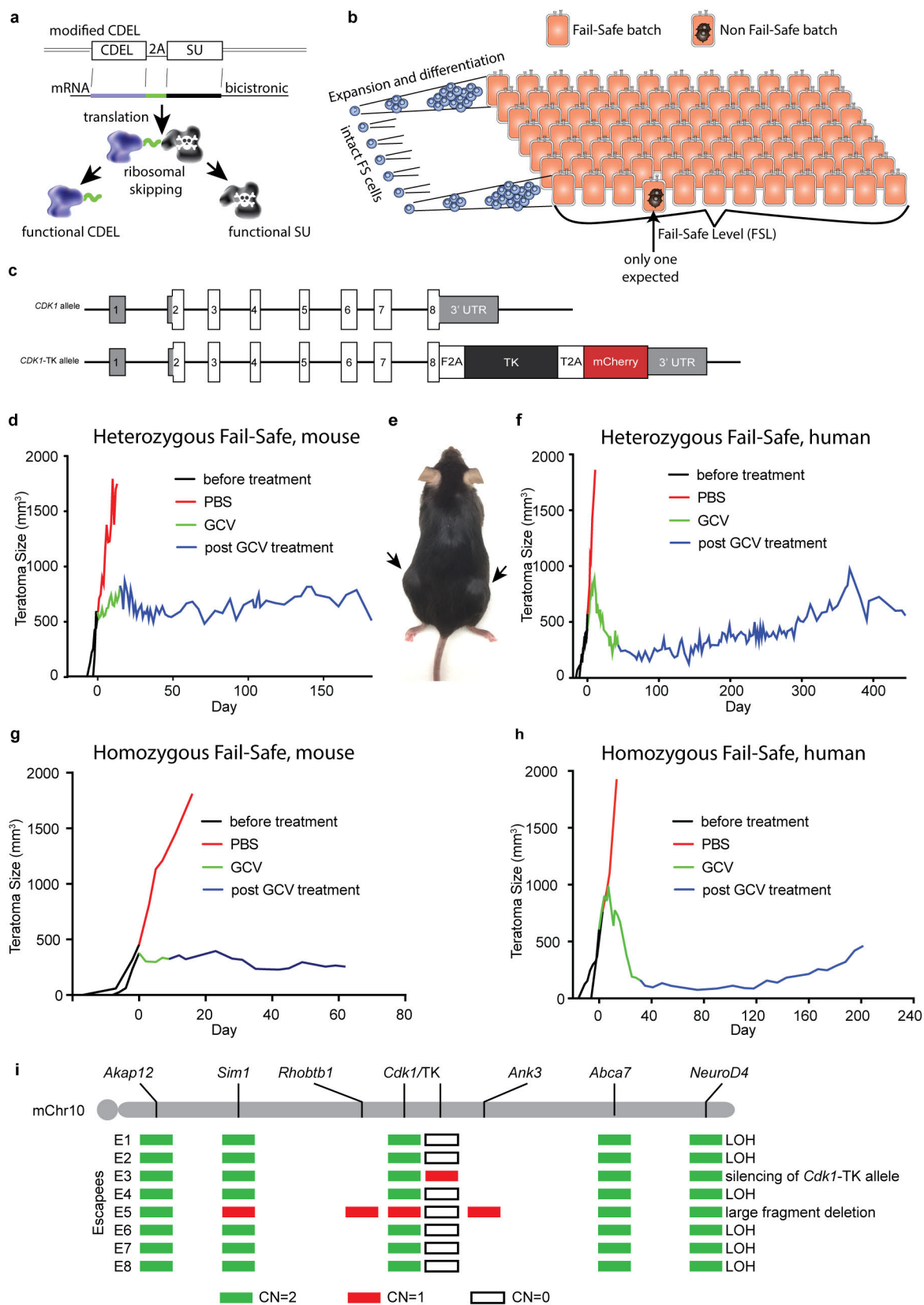
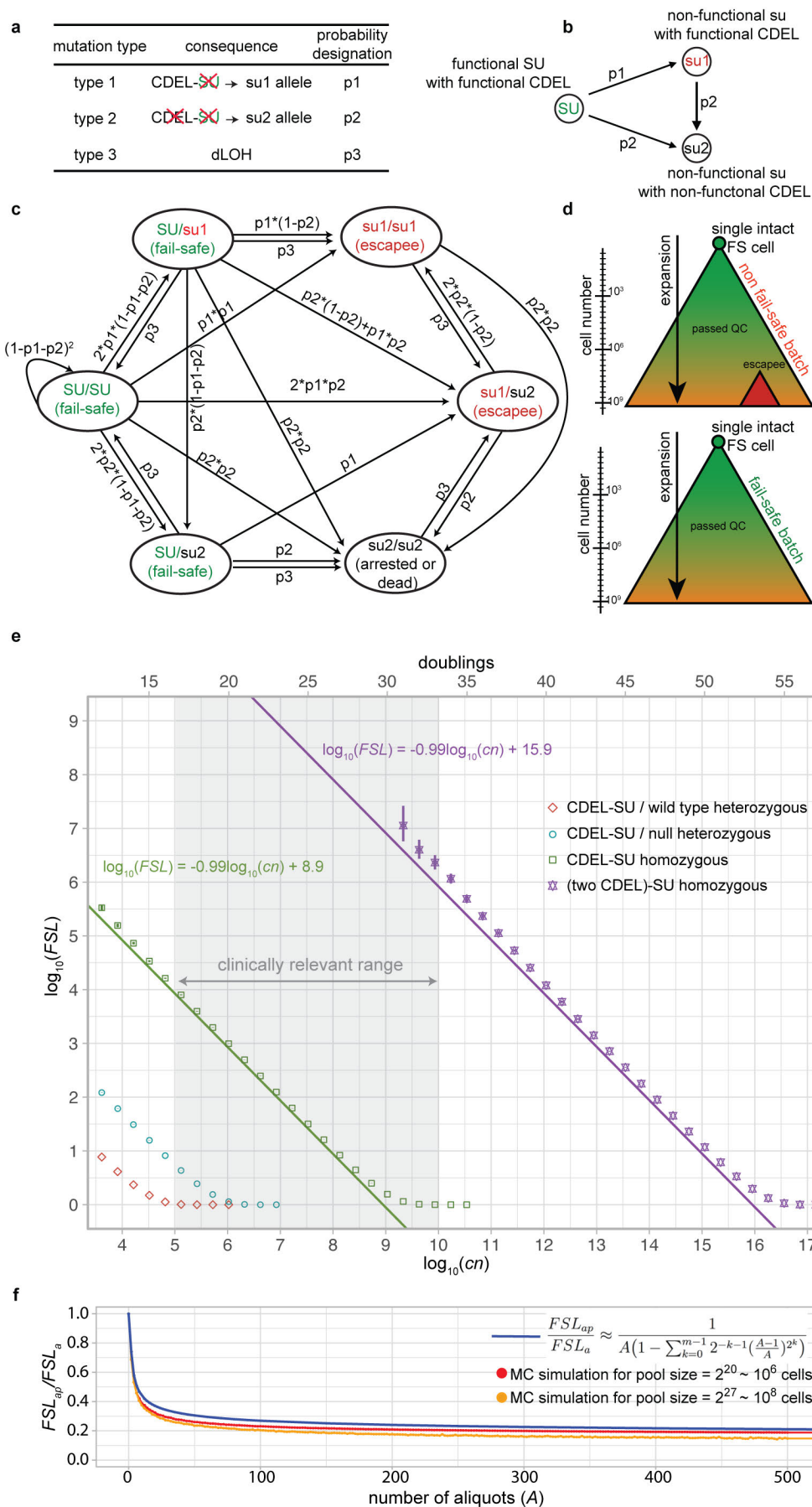
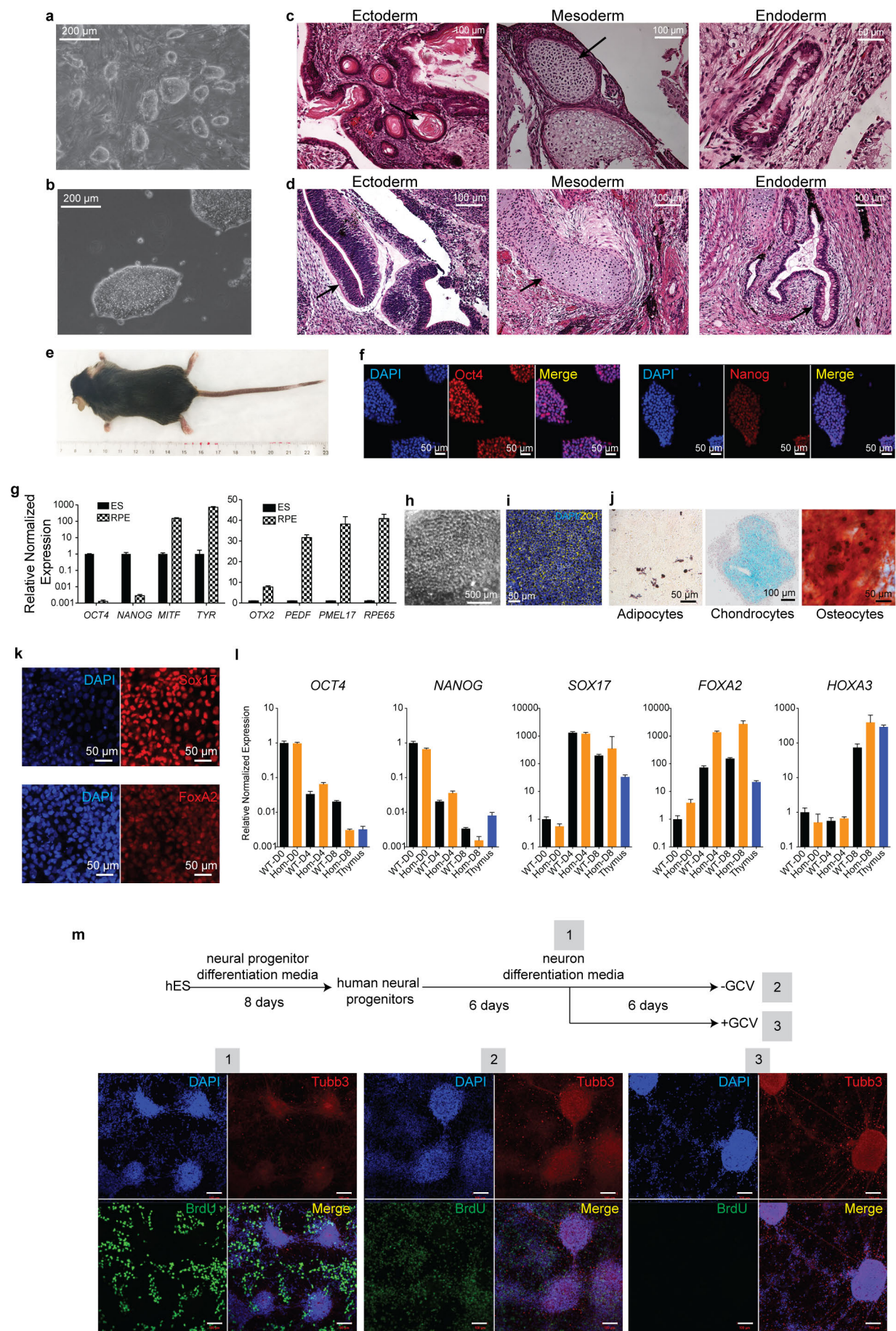


Figure 1 | Fail-safe cell system: the concept, the definition, realization and properties. **a**, The suicide gene is placed into a cell division essential locus (CDEL) resulting in a bicistronic mRNA that is translated into two proteins; a cell division essential factor and a drug-inducible suicide factor. **b**, Visual

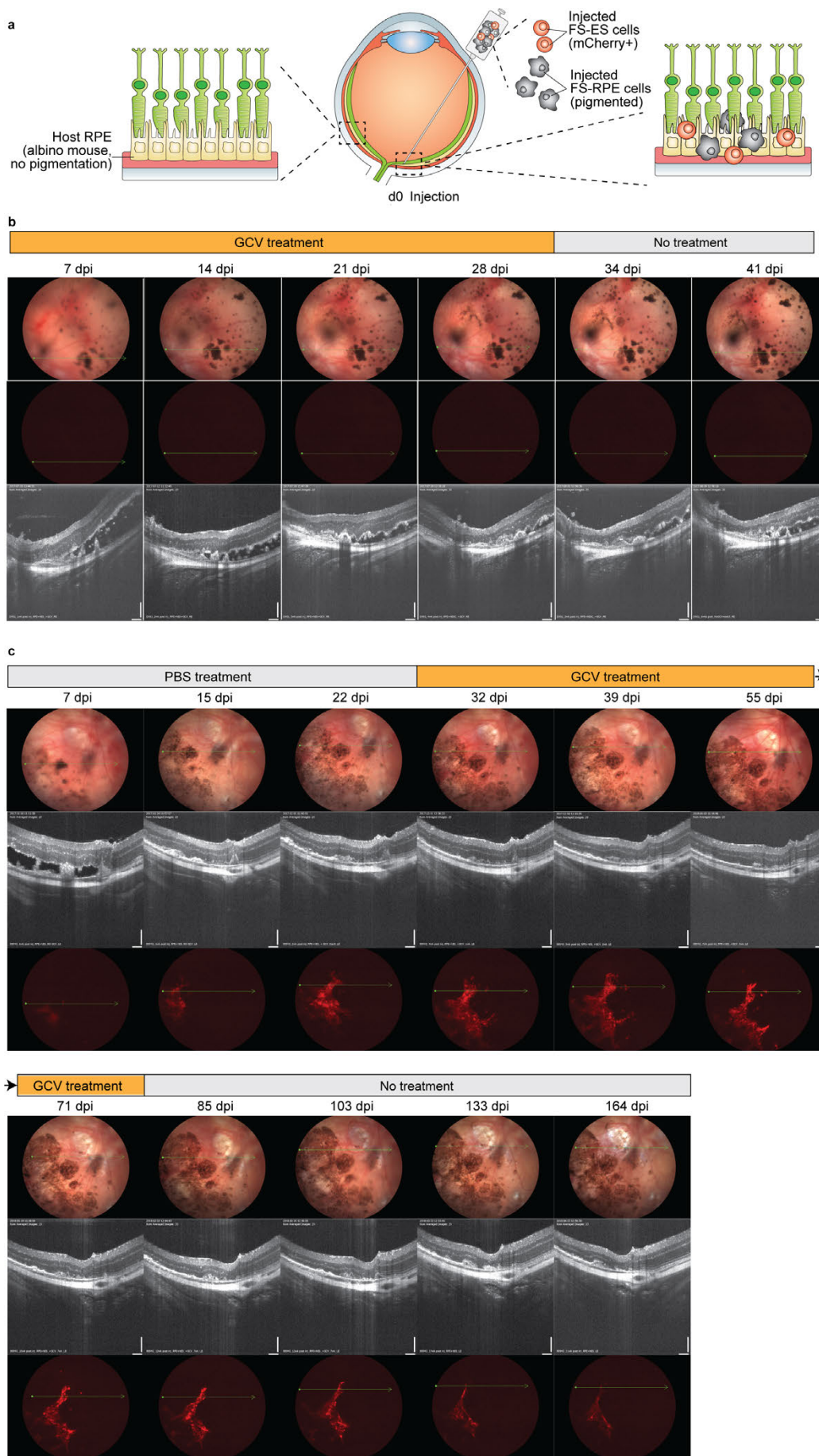
677 representation of the fail-safe level (FSL) defined by one non-fail-safe batch out of how many batches.
678 **c**, The link between the prototype SU (HSV-TK) and the prototype CDEL (*CDK1*). **d**, Representative
679 growth of teratomas formed by mouse *Cdk1*-TK/*Cdk1* cells, when the recipients were treated with PBS
680 or GCV. **e**, Adult mouse with stabilized subcutaneous tissue (fail-safe ES cell-derived dormant teratoma)
681 2.5 months after GCV treatment. **f**, Representative growth of teratomas formed by human *CDK1*-
682 TK/*CDK1* ES cells, when the recipients were treated with PBS or GCV. **g**, Representative growth of
683 teratomas formed by mouse *Cdk1*-TK/*Cdk1*-TK ES cells. **h**, Representative growth of teratomas formed
684 by human *CDK1*-TK/*CDK1*-TKES cells **i**, Copy number analysis of mouse *Cdk1*-TK/*Cdk1* escapees
685 identified in the experiment described in Extended Data Fig. 7.



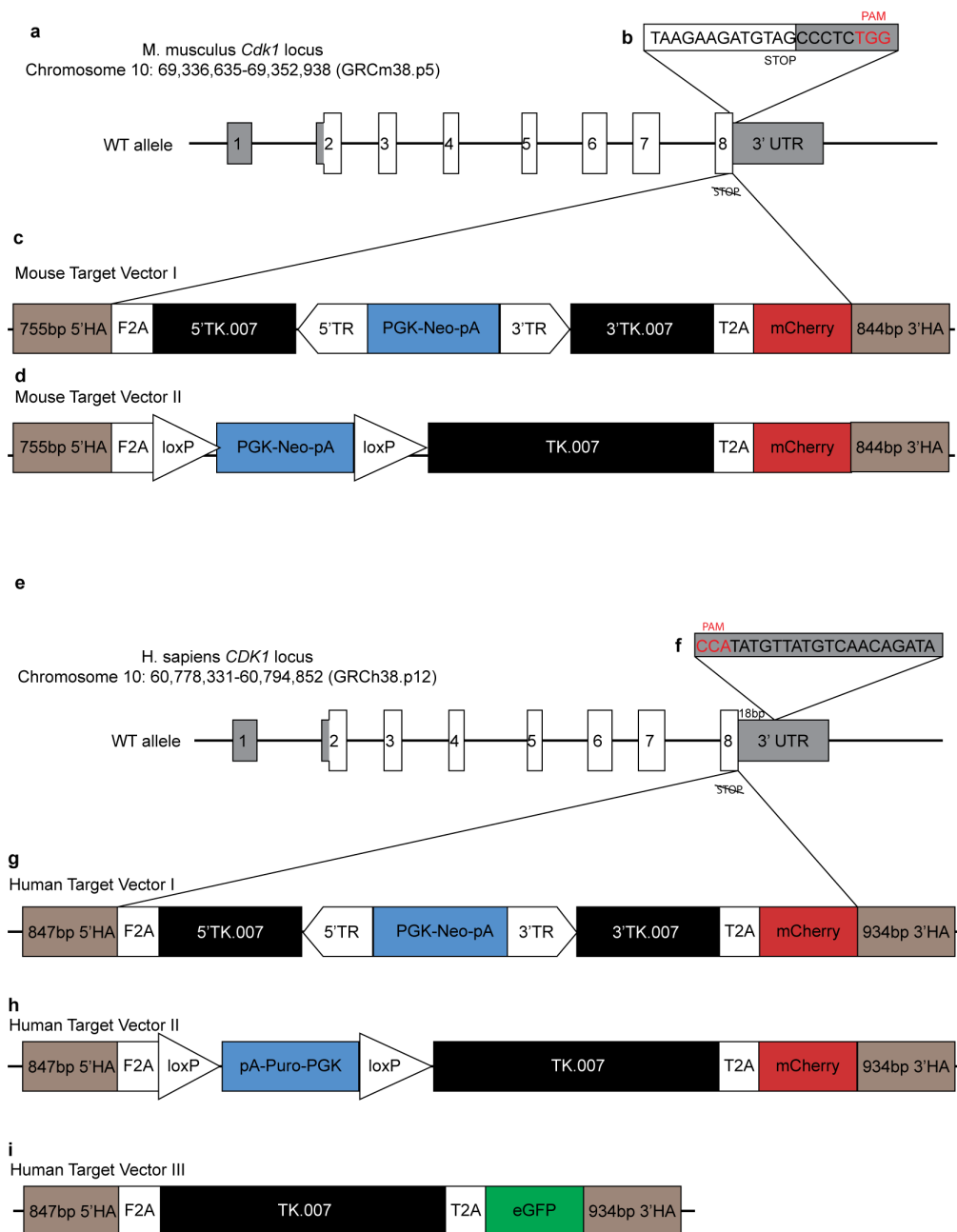
687 **Figure 2 | Modelling the FS cell system and calculating FSL.** **a**, Three types of mutations that could
688 affect the CDEL-SU allele. **b**, FS allele transition considered in the modelling and MC simulations. **c**,
689 Genotype transition matrix considered in the modelling and MC simulations. **d**, Visual illustration of FS
690 and non-FS batch formation during cell expansion. **e**, The function between therapeutic cell number and
691 FSL determined by MC simulation (data points) for different initiating cell genotypes. Solid lines show the
692 approximated linear regression on the “close to linear” part of the functions. The bars on certain data points
693 represent the 95% confidence intervals of the FSL estimates. **f**, The drop of FSL due to aliquoting from a
694 pool of cells relative to non-aliquoted batches of the same size.



696 **Figure 3 | Mouse and human fail-safe homozygous *CDK1*-TK/*CDK1*-TK cells demonstrate**
 697 **pluripotency.** All experiments were performed using the same clone of mouse C2 or human H1 (Exc16-
 698 3C) *CDK1*-TK/*CDK1*-TK cells. **a**, Bright-field photograph showing mouse homozygous *Cdk1*-
 699 TK/*Cdk1*-TK ES cell morphology. **b**, Bright-field photograph showing human homozygous *CDK1*-
 700 TK/*CDK1*-TK ES cell morphology. **c**, H&E staining of a mouse *Cdk1*-TK/*Cdk1*-TK ES cell derived
 701 teratoma. **d**, H&E staining of a human *CDK1*-TK/*CDK1*-TK ES cell derived teratoma. **e**, An adult *Cdk1*-
 702 TK/*Cdk1*-TK mouse. **f**, OCT4 and NANOG staining of human *CDK1*-TK/*CDK1*-TK ES cells. **g**, Q-PCR
 703 characterization of *CDK1*-TK/*CDK1*-TK ES cell differentiation into RPE cells. Values represent mean \pm
 704 SD, n = 3. **h**, Bright-field picture of human *CDK1*-TK/*CDK1*-TK ES cell derived RPE cells. **i**, ZO1
 705 staining of human *CDK1*-TK/*CDK1*-TK ES cell derived RPE cells. **j**, Human *CDK1*-TK/*CDK1*-TK ES
 706 cell derived adipocytes, chondrocytes and osteocytes. **k**, SOX17 and FOXA2 staining of human *CDK1*-
 707 TK/*CDK1*-TK ES cell derived definitive endoderm. **l**, *OCT4*, *NANOG*, *SOX17*, *FOXA2* and *HOXA3* Q-
 708 PCR characterization of ES cell (Day 0) differentiation into definitive endoderm (Day 4) and pharyngeal
 709 pouch endoderm (Day 8). Values represent mean \pm SD, n = 3. **m**, Human *CDK1*-TK/*CDK1*-TK ES cell
 710 differentiation into neural epithelial progenitors and subsequent neurons, with or without GCV
 711 treatment. Scale bar 100 μ m.

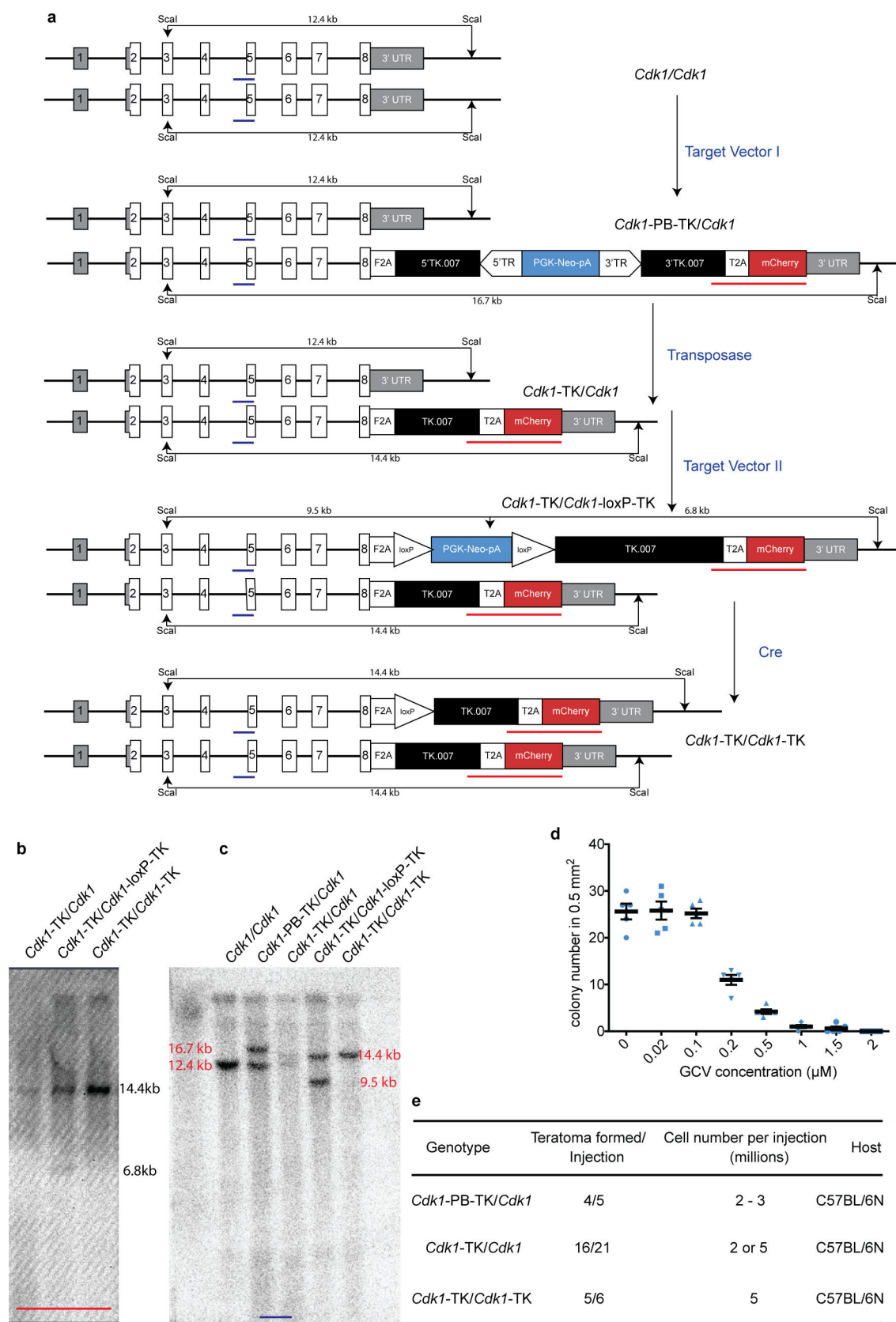


713 **Figure 4 | Fail-safe cell system in action; an *in vivo* proof of principle study.** **a**, A 3:1 mixture of
714 human homozygous FS ES cell-derived RPE cells and homozygous FS ES cells were subretinally co-
715 injected into NSG mice and imaged using fundoscopy and optical coherence tomography (OCT)
716 throughout GCV or PBS treatment. **b**, Fundoscopy, OCT and fluorescence imaging of the eye receiving
717 GCV treatment (4 weeks). **c**, Fundoscopy, OCT and fluorescence imaging of the eye of the mouse that
718 received PBS treatment (3 weeks) and developed an actively growing ES cell-derived lesion (mCherry+
719 cells). dpi stands for days post injection.



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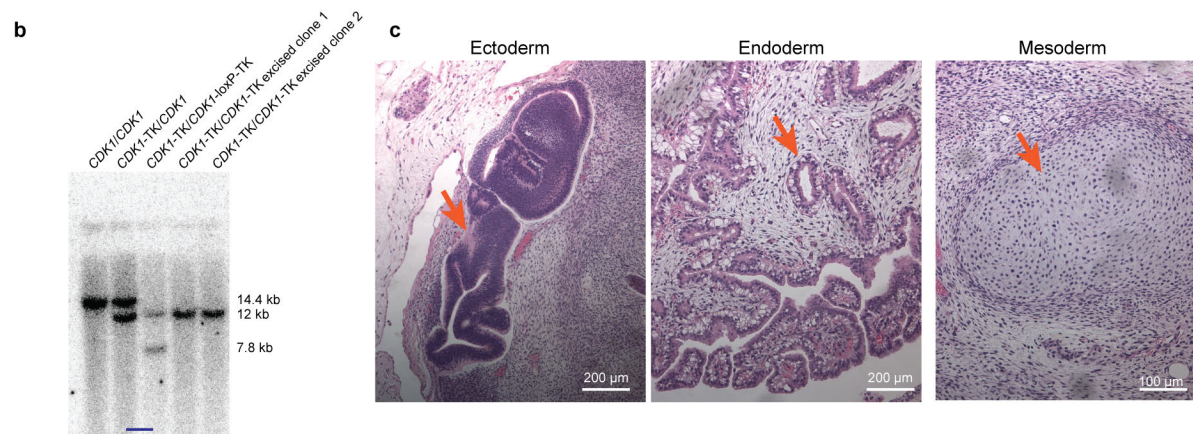
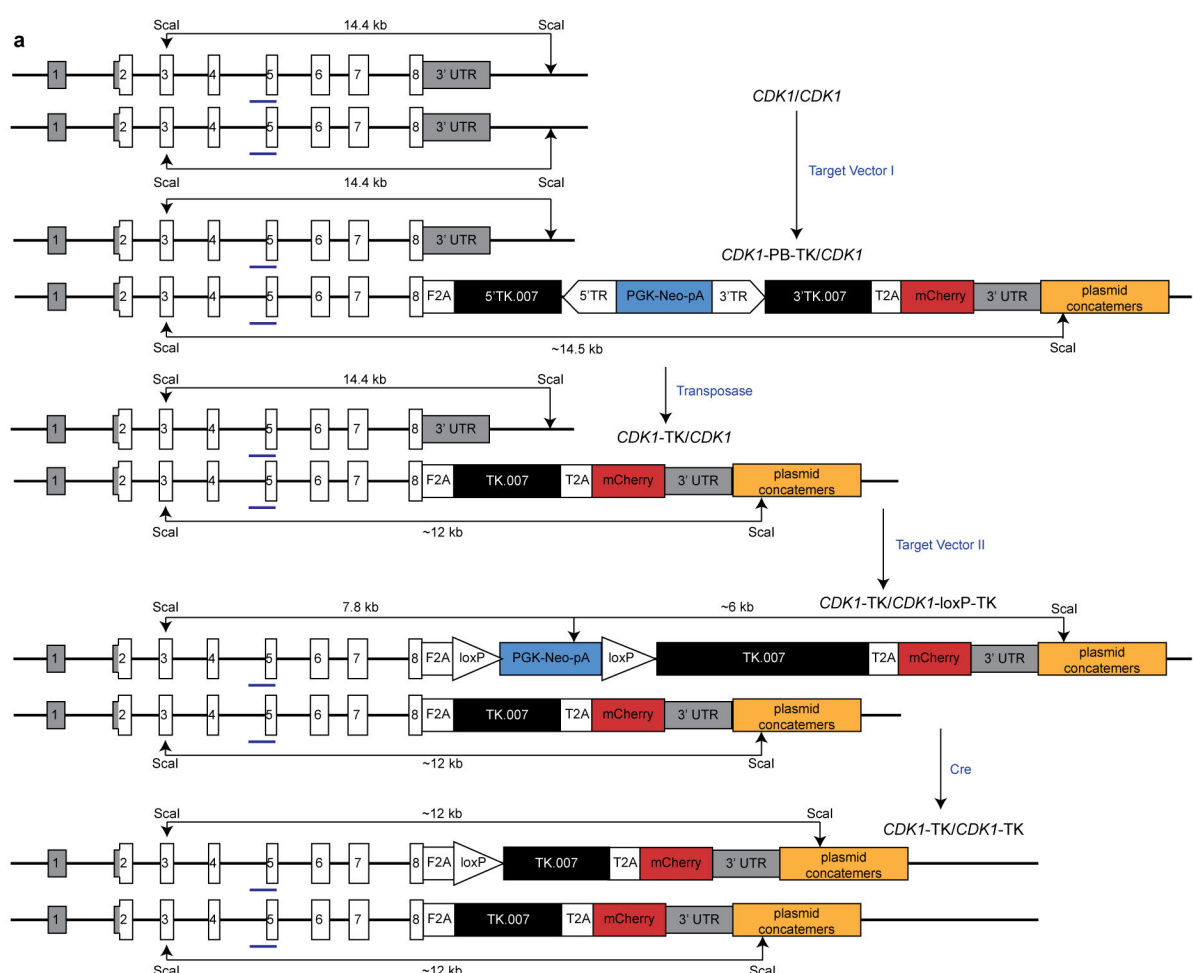
722 **Extended Data Figure 1 | Schematic of targeting vectors for mouse and human *CDK1*.** **a, e,** The
723 genomic locus and structure of the mouse (**a**) and human (**e**) *CDK1* gene. **b, f,** CRISPR/Cas9 target site for
724 mouse (**b**) and human (**f**) *CDK1*. **c, d, g, h, i,** The targeting vectors used in the study. The knock-in
725 insertion replaced the *CDK1* stop codon with an F2A sequence, followed by the TK.007 linked to a
726 fluorescent protein gene with a T2A sequence. Adding fluorescent reporters (mCherry and eGFP) to the
727 insert permitted the visualisation of the modified *CDK1* allele expression in the cells. A positive selectable
728 marker (neomycin or puromycin) was used to select for integration, and was subsequently removed by
729 transiently expressing Cre recombinase or piggyBac (PB) transposase in the targeted cells, depending on
730 the flanking loxP or PB terminal repeats (TR) in the target vector type, respectively. UTR: untranslated
731 region, HA: homology arm, PGK: phosphoglycerate kinase, eGFP: enhanced green fluorescent protein,
732 mCherry: mCherry fluorescent protein, pA: polyadenylation, Neo: neomycin selectable marker, Puro:
733 puromycin selectable marker.



Extended Data Figure 2 | Generation, genotyping and characterization of mouse C57BL/6N C2 *Cdk1*-TK/*Cdk1* and *Cdk1*-TK/*Cdk1*-TK ES cells. a, Summary of the targeting steps used to generate

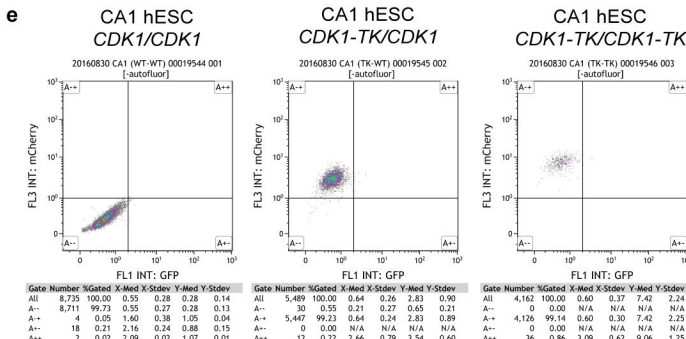
738 mouse C2 *Cdk1*-TK/*Cdk1* and *Cdk1*-TK/*Cdk1*-TK ES cells. **b**, Southern blot genotyping with internal
739 TK-mCherry probe. **c**, Southern blot genotyping with mouse *Cdk1* genomic probe. **d**, *In vitro* GCV
740 dose-response killing curve of mouse C2 *Cdk1*-TK/*Cdk1* ES cells. **e**, Teratoma formation efficiency of
741 mouse C2 *Cdk1*-PB-TK/*Cdk1*, *Cdk1*-TK/*Cdk1* and *Cdk1*-TK/*Cdk1*-TK ES cells.

743 **Extended Data Figure 3 | Generation, genotyping and characterization of human H1 *CDK1*-**
744 **TK/*CDK1* and *CDK1*-TK/*CDK1*-TK ES cells. a,** Steps of generating human H1 *CDK1*-TK/*CDK1* and
745 *CDK1*-TK/*CDK1*-TK ES cells. **b,** Southern blot genotyping of the *CDK1*-TK/*CDK1* clone Exc16 used
746 in teratoma assays (Fig. 1c and Extended Data Fig. 7) and the *CDK1*-TK/*CDK1*-TK clone Exc16-3C
747 used in the differentiation assays in Fig. 3. **c,** PCR genotyping of all the correct clones. **d,** Flow
748 cytometry analysis of the *CDK1*-TK/*CDK1* clone Exc16 and the *CDK1*-TK/*CDK1*-TK clone Exc16-3C.
749 **e,** SybrGreen Q-PCR of human *CDK1* expression in H1 WT, the *CDK1*-TK/*CDK1* clone Exc16 and the
750 *CDK1*-TK/*CDK1*-TK clone Exc16-3C. Values represent mean \pm SD, n = 3. No significant difference
751 among them. **f,** TaqMan Q-PCR copy number analysis of TKs of all the clones with the correct
752 genotype. **g,** Table showing the efficiency of teratoma formation in NSG mice using H1 human ES cells.
753 **h,** Dose-response analysis of wild type, *CDK1*-TK/*CDK1* and *CDK1*-TK/*CDK1*-TK human H1 ES cells.
754 Cells were treated with different GCV concentrations, dissociated and counted after 7 days.

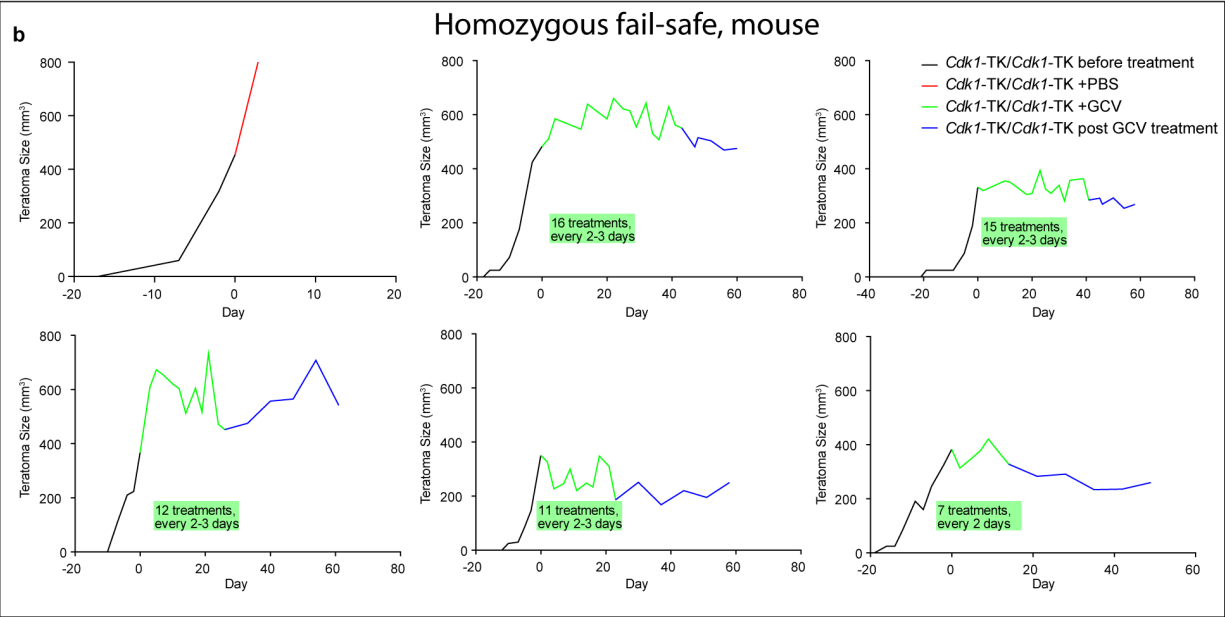
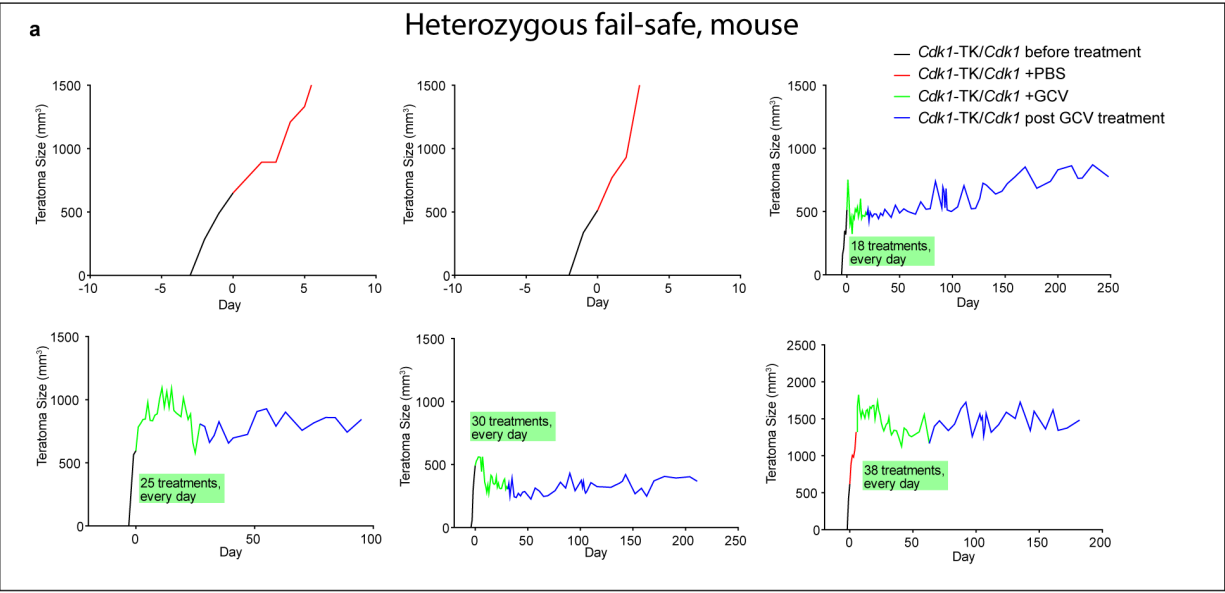


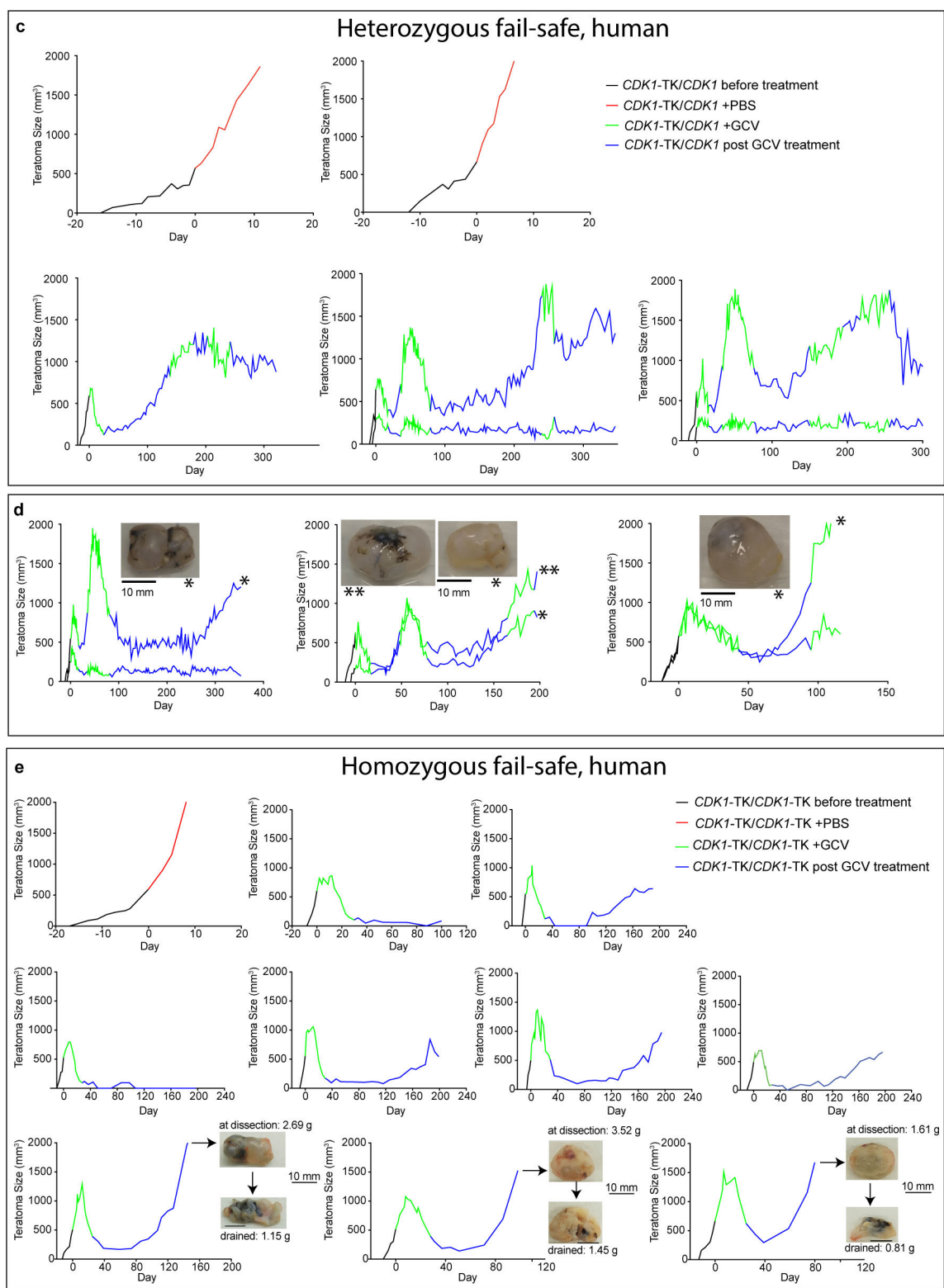
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Genotype	Teratoma formed/ Injections	Cell number per injection	Host
CDK1-TK/CDK1-TK	4/4	1 - 4 x 10 ⁶	NSG



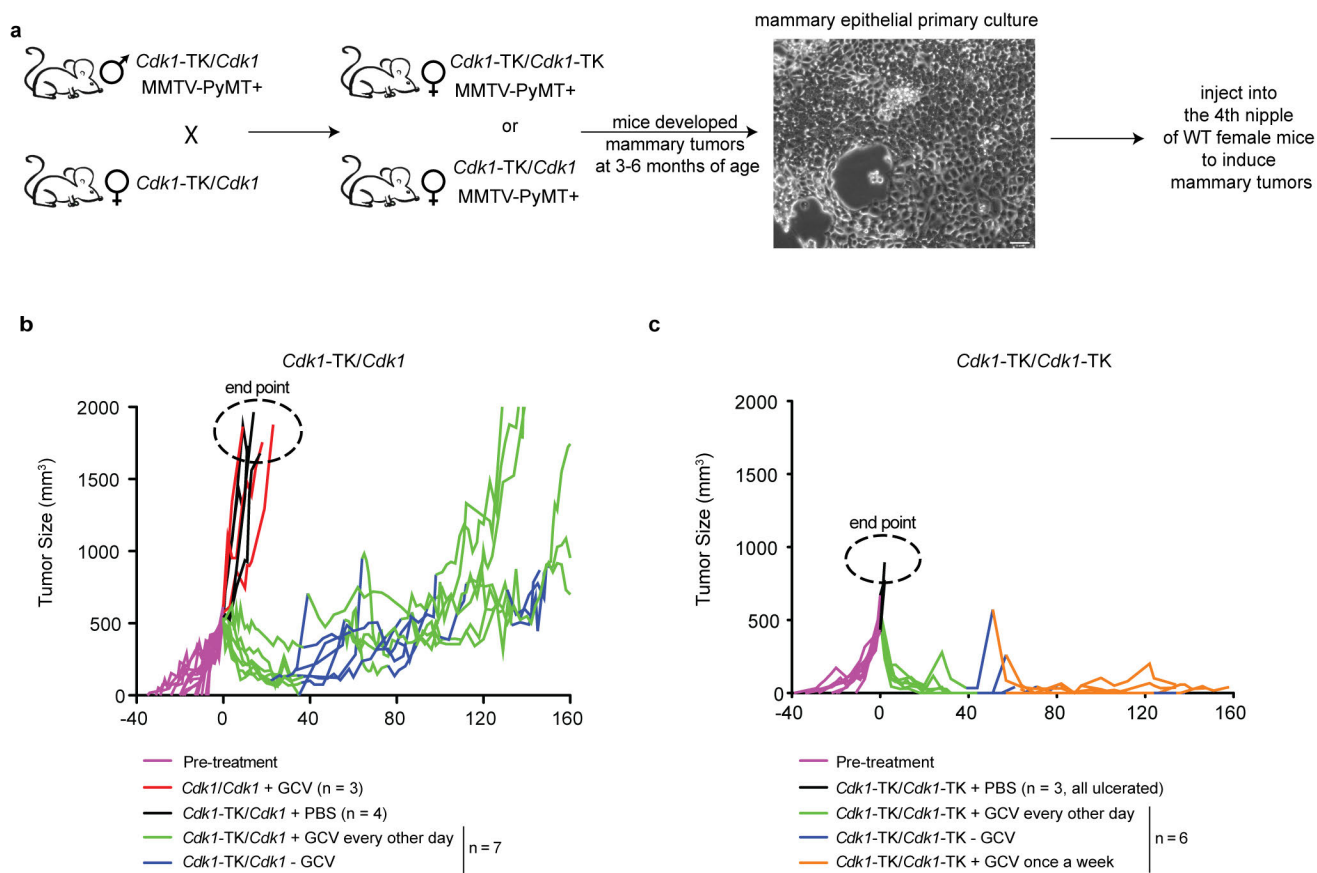
756 **Extended Data Figure 4 | Generation, genotyping and characterization of human CA1 *CDK1*-**
757 **TK/*CDK1* and *CDK1*-TK/*CDK1*-TK ES cells. a,** Steps of generating human CA1 *CDK1*-TK/*CDK1*
758 and *CDK1*-TK/*CDK1*-TK ES cells. **b,** Southern blot genotyping of human CA1 *CDK1*-TK/*CDK1* and
759 *CDK1*-TK/*CDK1*-TK ES cells. The plasmid concatemers are multiple copies of plasmid integration
760 (including backbone). The Ampicillin gene in the backbone contains a *ScaI* restriction enzyme site
761 which is consistent with the sizes of the band in southern blots. **c,** H&E staining of a *CDK1*-TK/*CDK1*-
762 TK CA1 ES cell derived teratoma.





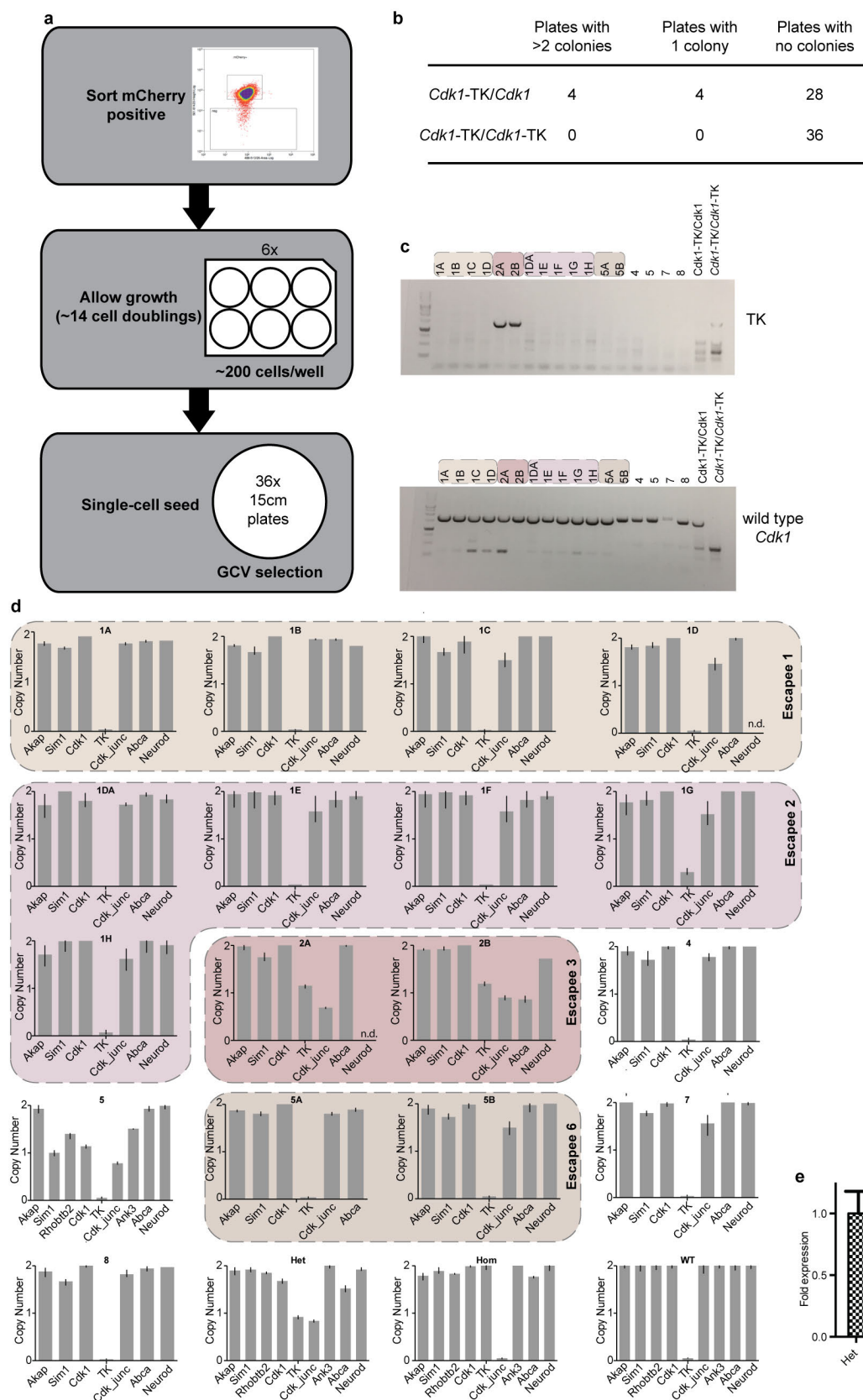
Extended Data Figure 5 | Growth graphs of mouse and human ES cells-derived teratomas. a, Growth of teratomas derived from mouse heterozygous fail-safe ES cells (*C2 Cdk1-TK/Cdk1*) **b,** Growth of teratomas derived from mouse homozygous fail-safe ES cells (*C2 Cdk1-TK/Cdk1-TK*) **c,** Growth of teratomas derived from human heterozygous fail-safe ES cells (H1 *CDK1-TK/CDK1*, clone Exc16), daily GCV treatment. **d,** Examples of teratomas from human heterozygous fail-safe ES cells

771 showing cyst formation, images of cystic teratomas at dissection are shown next to the corresponding
772 growth line, daily GCV treatment. The graphs with two lines represent mice that had cells injected to
773 both flanks. The graphs with one line represent mice that had cells injected to one flank. The GCV
774 treatment regime varies among mice because each teratoma behaves differently; we started GCV when
775 the teratoma size started to increase. **e**, Growth of teratomas derived from human homozygous fail-safe
776 ES cells (H1 *CDK1*-TK/*CDK1*-TK), GCV treatment was every other day. Images of cystic teratomas are
777 shown next to the corresponding growth line, cysts were drained after dissection to show the difference
778 in tumour weight due to the fluid present in the tissue. Each graph represents one mouse.



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781 **Extended Data Figure 6 | Breast cancer transplantation assay using heterozygous and homozygous**
 782 **FS mammary tumour cells. a**, Generation of mouse line and experimental design. **b**, Growth of
 783 mammary gland tumours derived from mouse *Cdk1/Cdk1* and *Cdk1-TK/Cdk1* mammary epithelial cells
 784 with PBS or GCV treatment. **c**, Growth of mammary gland tumours derived from mouse *Cdk1-TK/Cdk1-*
 785 *TK* mammary epithelial cells with PBS or GCV treatment.



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788 **Extended Data Figure 7 | *In vitro* experiments with mouse *Cdk1-TK/Cdk1*, *Cdk1-TK/Cdk1-TK* ES**
789 **cells and subsequent characterization of escapees. a**, Experimental design: mCherry+ cells were

790 selected by sorting to ensure that the starting cell population did not contain escapees. These cells were
791 plated on 6 well plates (200 cells/well, 36 total wells) and allowed to grow to 14 cell doublings (this was
792 estimated by counting cells in sample wells). The 36 cultures were then resuspended to a single-cell
793 suspension and each was plated to a 15 cm plate (4×10^6 cells). One day after plating, selection with GCV
794 was started and maintained until escapee colonies appeared. **b**, Escapee numbers obtained in 36
795 independent cultures growing from *Cdk1*-TK/*Cdk1*, *Cdk1*-TK/*Cdk1*-TK ES cells. **c**, PCR to determine the
796 presence of TK. **d**, TaqMan copy number Q-PCR analysis of *Akap*, *Sim1*, *Cdk1* junction of exon 8 and
797 3'UTR, *Neurod*, *Cdk1*, *TK*, *Abca* on mouse Chr. 10. Values represent the copy number calculated by
798 CopyCaller Software v2.1 and the bars indicate the range from the minimum to the maximum number. n =
799 3. The same color in the background of **c** and **d** indicates that they are from the same independent culture.
800 **e**, Q-PCR to compare TK expression level in *Cdk1*-TK/*Cdk1* escapee clone 2A and C2 WT ES cells.
801 Values represent mean \pm SEM. n = 3.

a

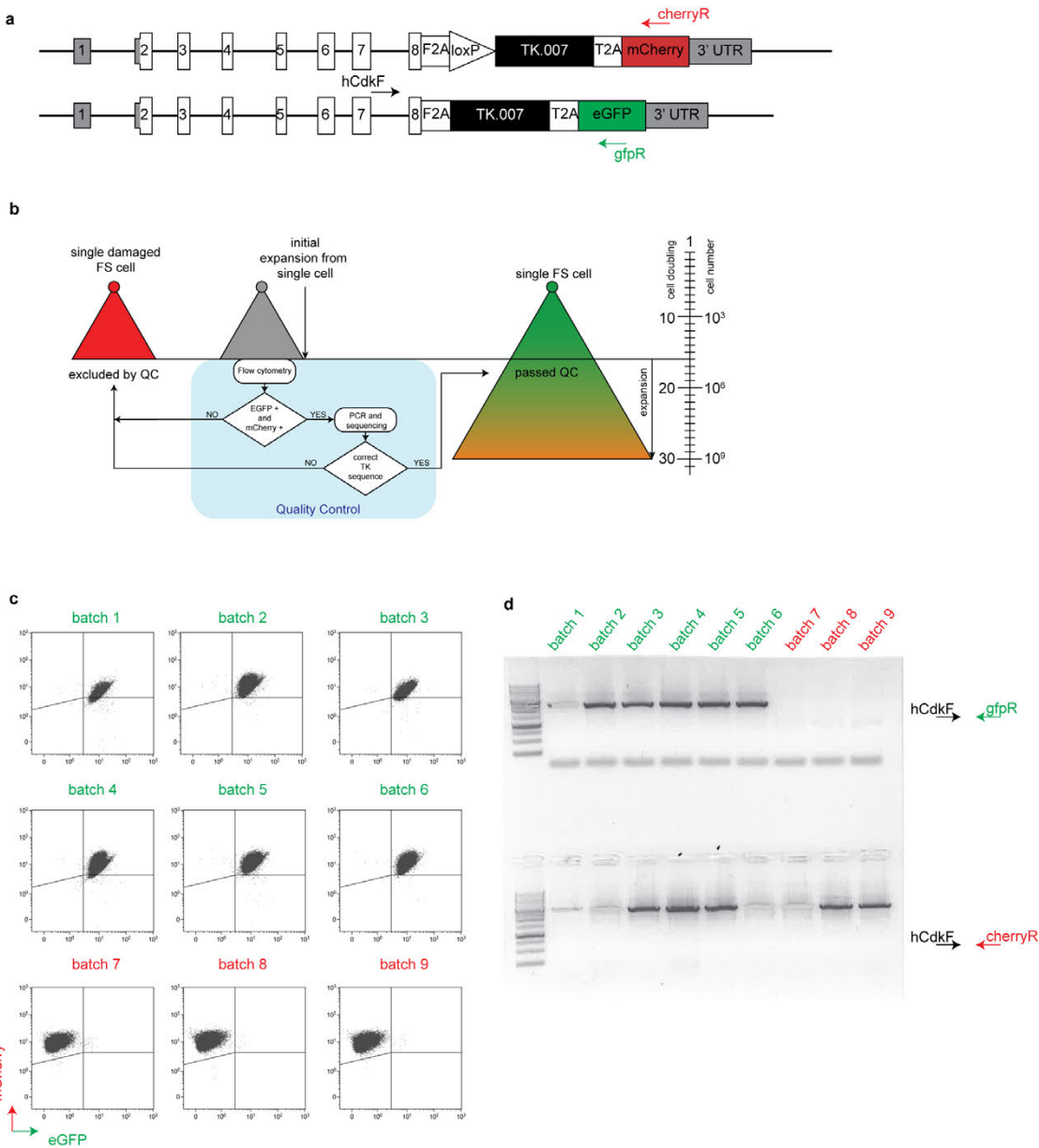
Number of replicate culture	Initial number of cells per culture	Average number of cells/ culture	Total numbers of cells	Total live cells analyzed	Live cells / total cells	Average mutants per sample	LOH rate per cell division
21	1	4.9x10 ⁶	4.9x10 ⁶ x21=1.029x10 ⁸	18,833,305	0.18	eGFP only: 303 mCherry only: 251	mCherry loss frequency: 9.05x10 ⁻⁶ eGFP loss frequency: 7.68x10 ⁻⁶

b

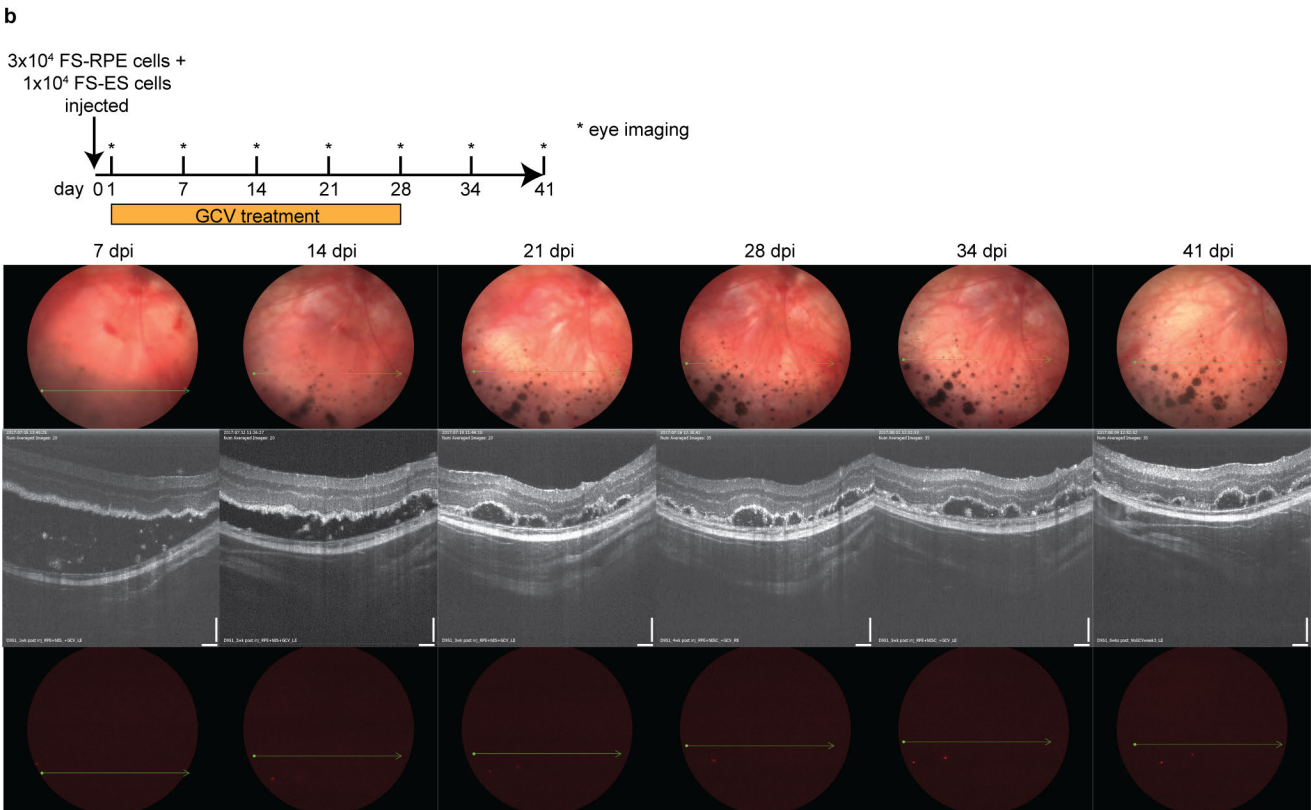
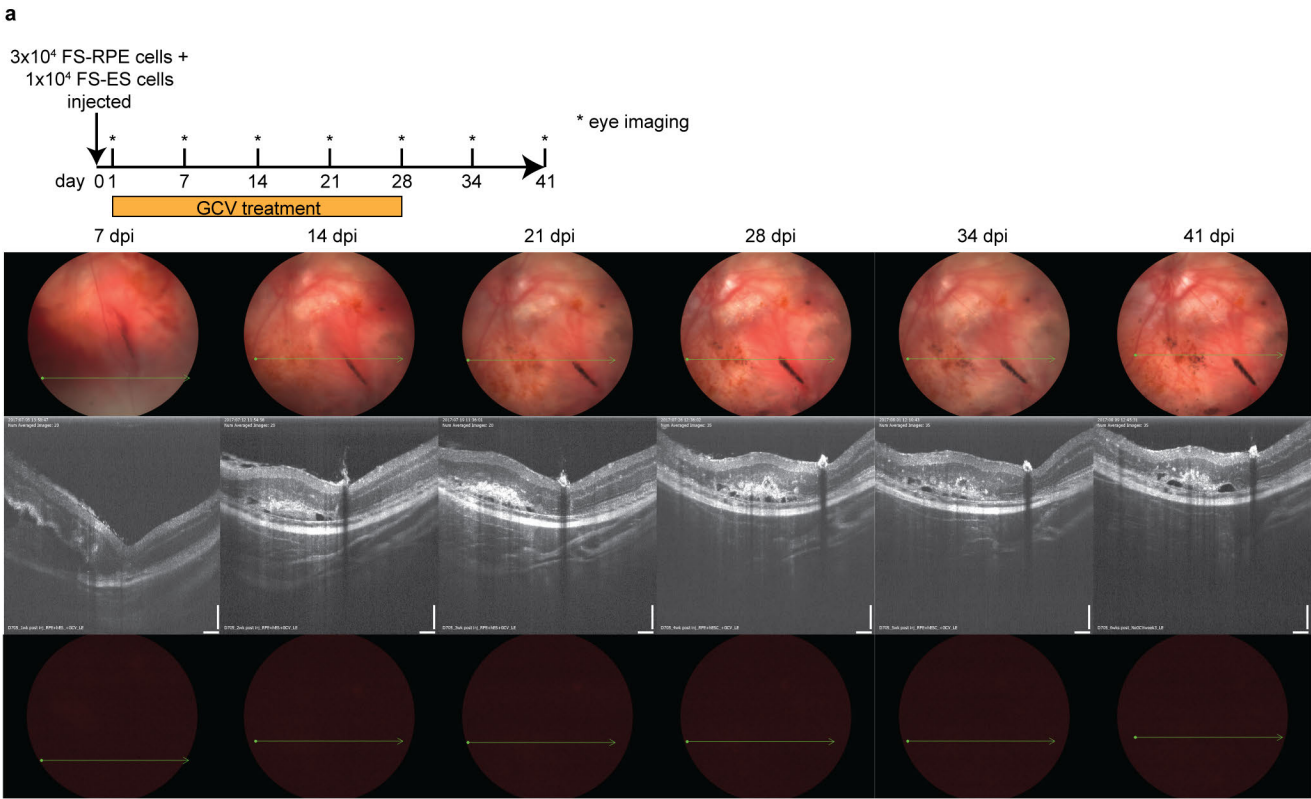
Samples	All events	Live cells	Single cells	eGFP+ mCherry-	mCherry+ eGFP-
1	50,000	36,102	14,261	0	1
2	1,387,922	1,166,927	663,704	11	32
3	1,306,685	1,229,940	734,030	59	95
4	1,977,744	1,473,607	1,229,131	29	38
5	2,492,764	2,051,819	1,798,915	114	114
6	2,639,618	2,160,508	1,891,409	102	108
7	559,819	406,149	374,528	18	3
8	39,161	15,380	12,977	0	1
9	1,661,726	1,354,148	1,153,558	156	81
10	2,315,402	1,911,681	1,664,292	63	75
11	843,549	611,761	529,324	124	45
12	2,088,898	1,595,412	1,337,789	86	69
13	2,468,994	1,853,728	1,628,365	69	75
14	92,019	56,795	46,791	10	9
15	1,791,662	1,076,661	953,464	7	4
16	2,153,934	1,680,018	1,485,732	179	106
17	1,416,523	1,029,636	882,532	7	8
18	1,096,211	771,314	653,076	32	25
19	15,695	8,297	6,665	1	0
20	764,311	598,206	533,928	28	6
21	1,802,185	1,369,832	1,238,834	72	73
Total	28,964,822	22,457,921	18,833,305	1,167	968
Total mutants in all samples				6,376.16	5,288.89
Average mutants per sample				303.62	251.85
Mutation rate				9.05x10 ⁻⁶	7.68x10 ⁻⁶

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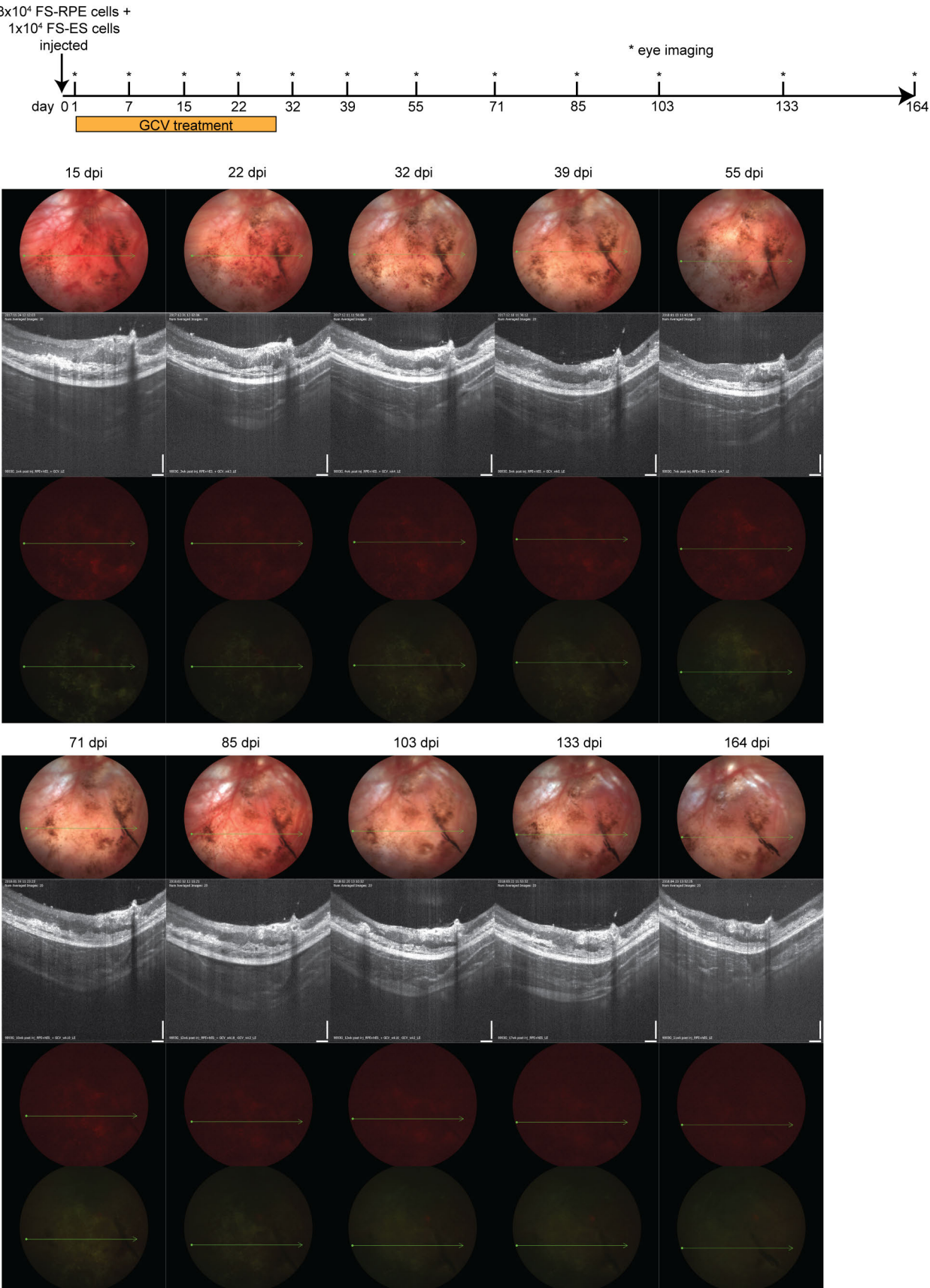
804 **Extended Data Figure 8 | Calculation of the LOH rate at the human *CDK1* locus with human H1**
805 ***CDK1*-TK/*CDK1*-TK ES cells. a, Summary table. b, Detailed cell number for each individual clone.**



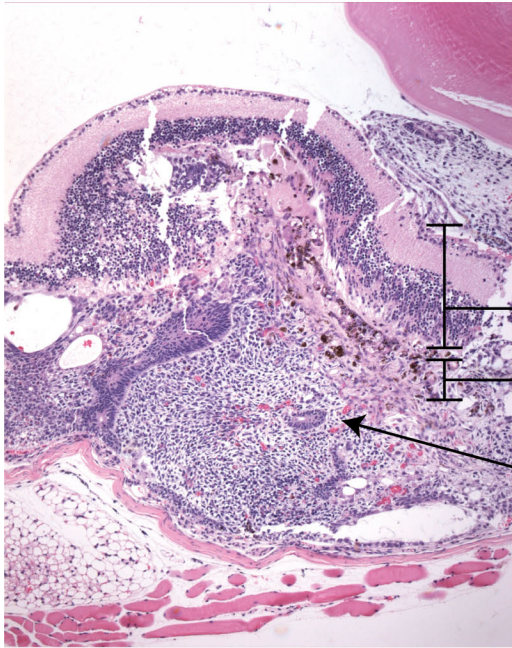
Extended Data Figure 9 | Quality control (QC) of batches generated from single human H1 *CDK1*-TK/*CDK1*-TK ES cells. **a**, Schematics of the alleles in the *CDK1*-TK/*CDK1*-TK human ES cells used in the quality control. **b**, Workflow schematic of performing QC on several ES cell batches. **c**, An example of the flow cytometry for the QC of nine clonally derived batches. **d**, An example of PCR for the QC of nine clonally derived batches.



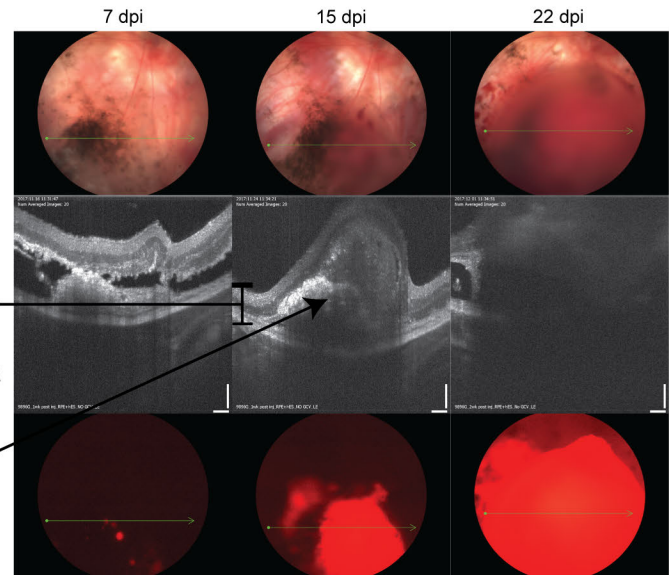
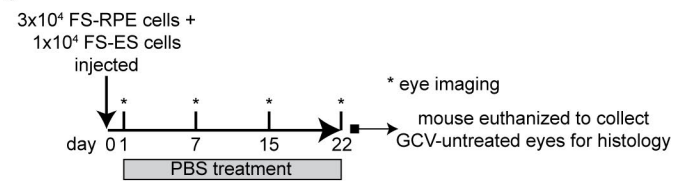
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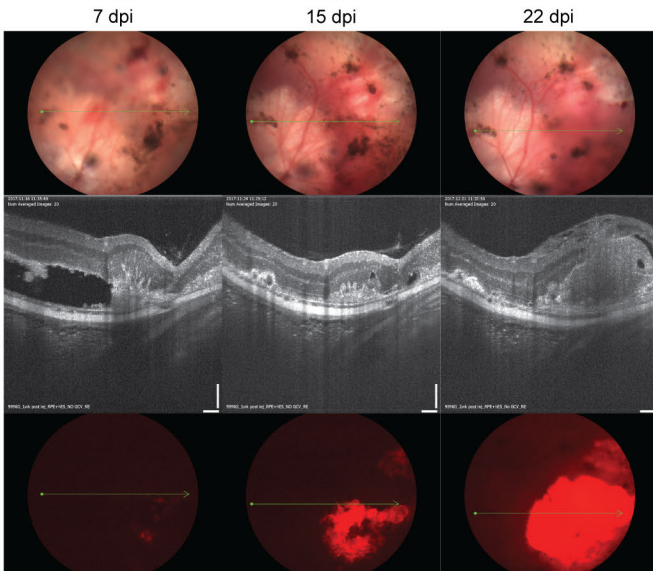
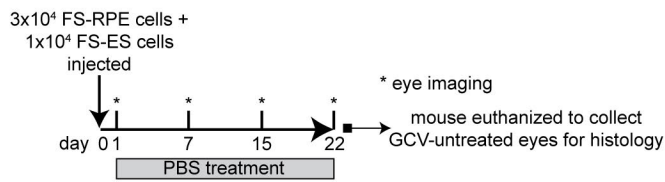
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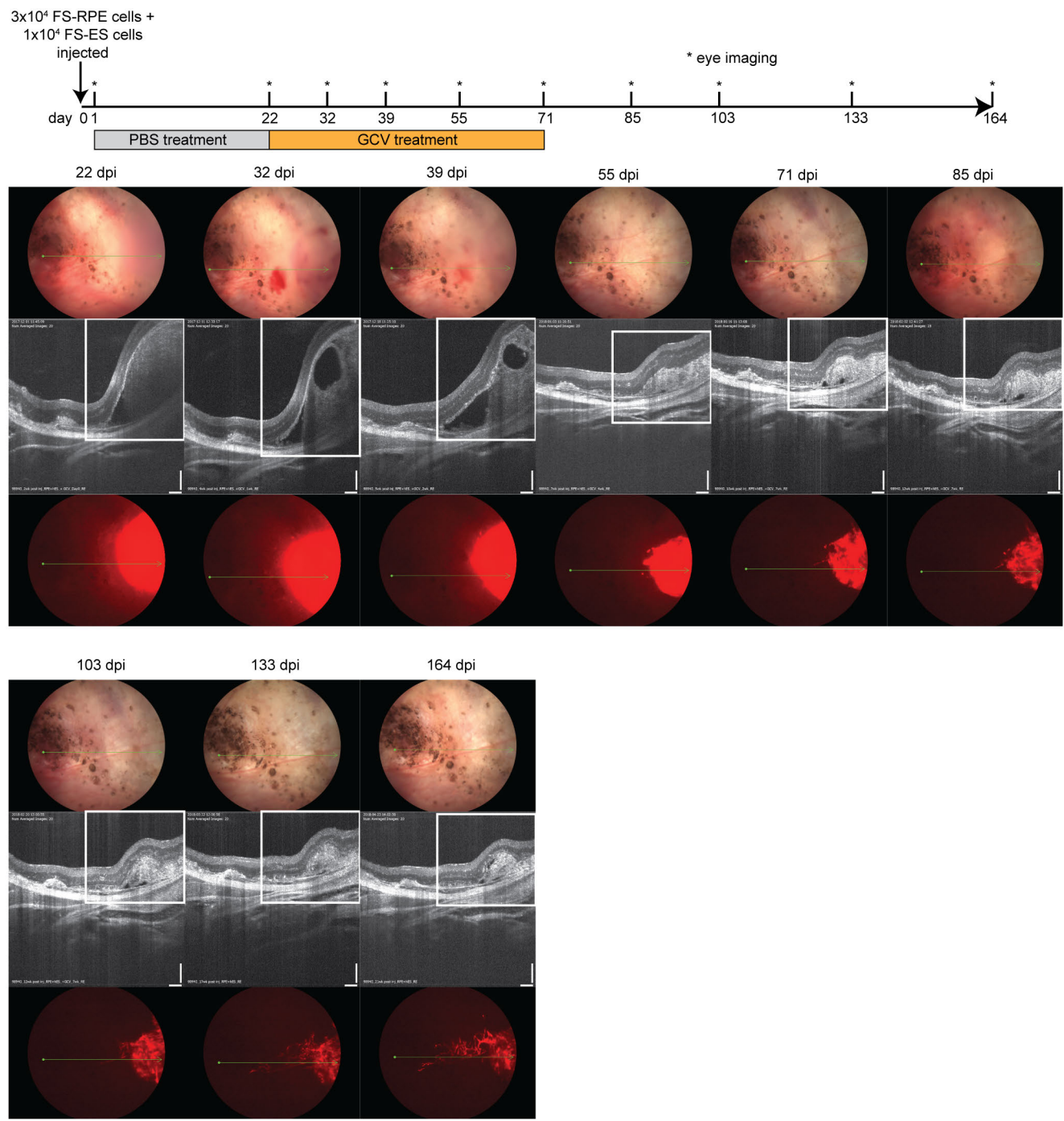
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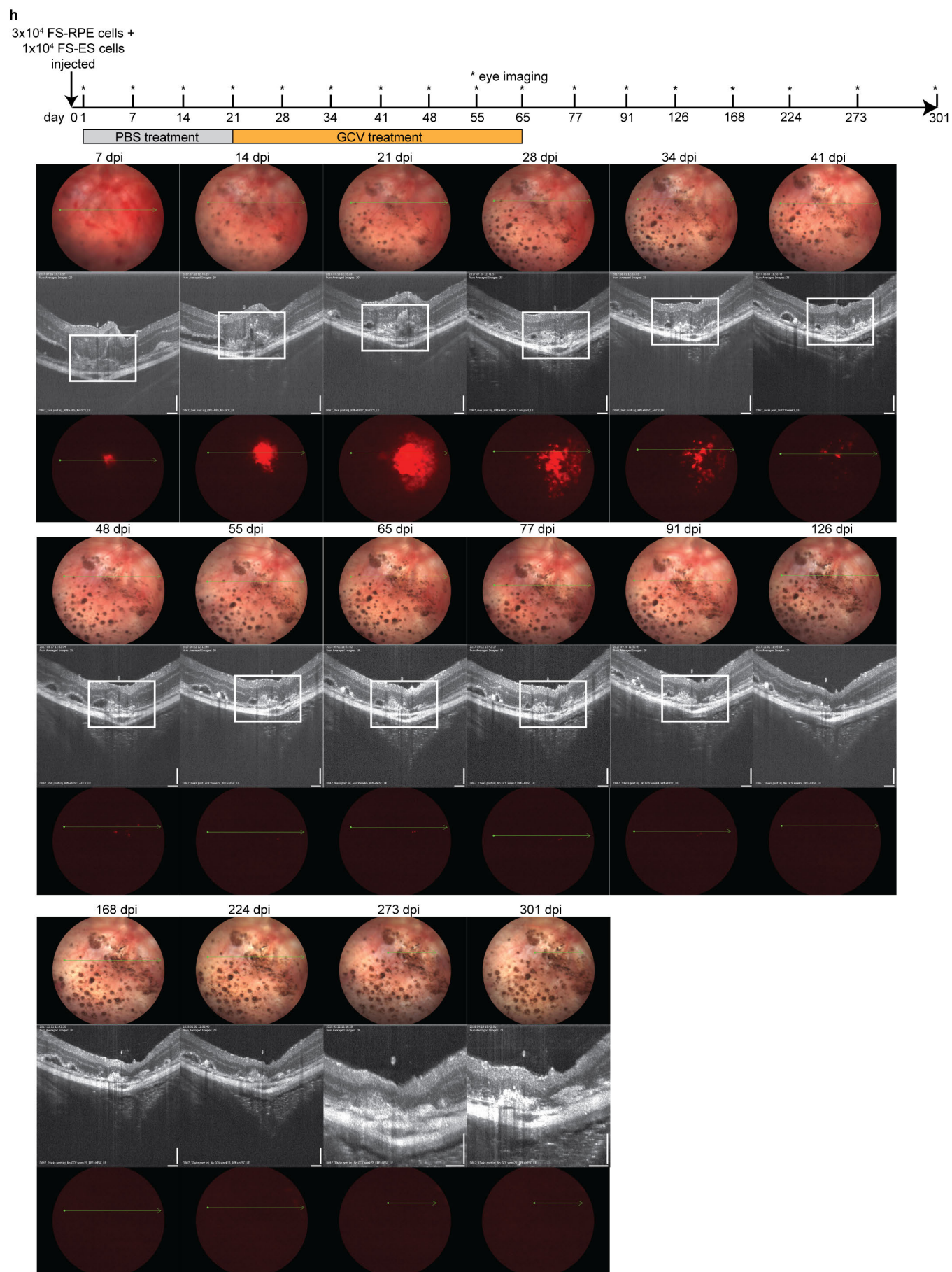


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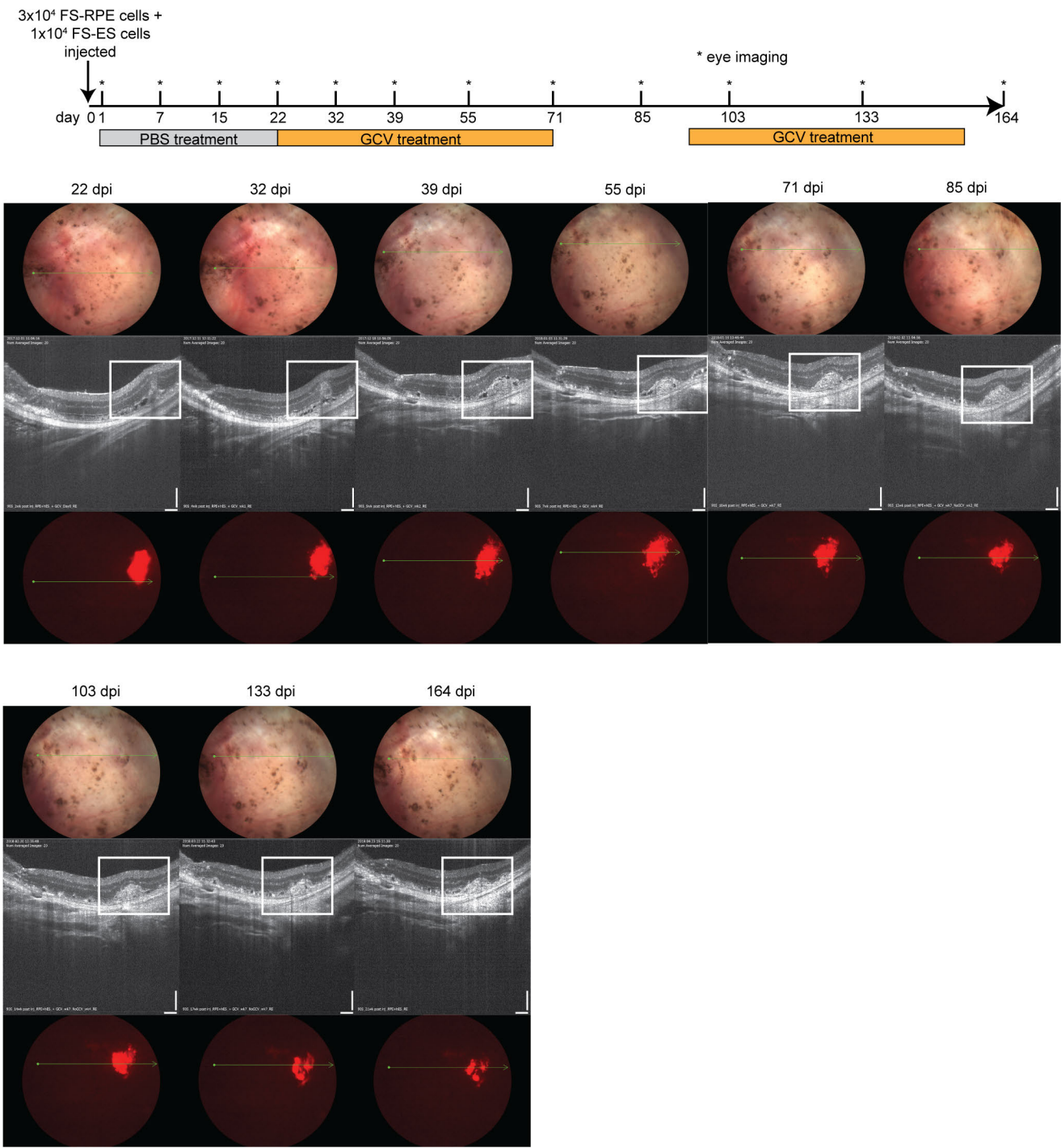


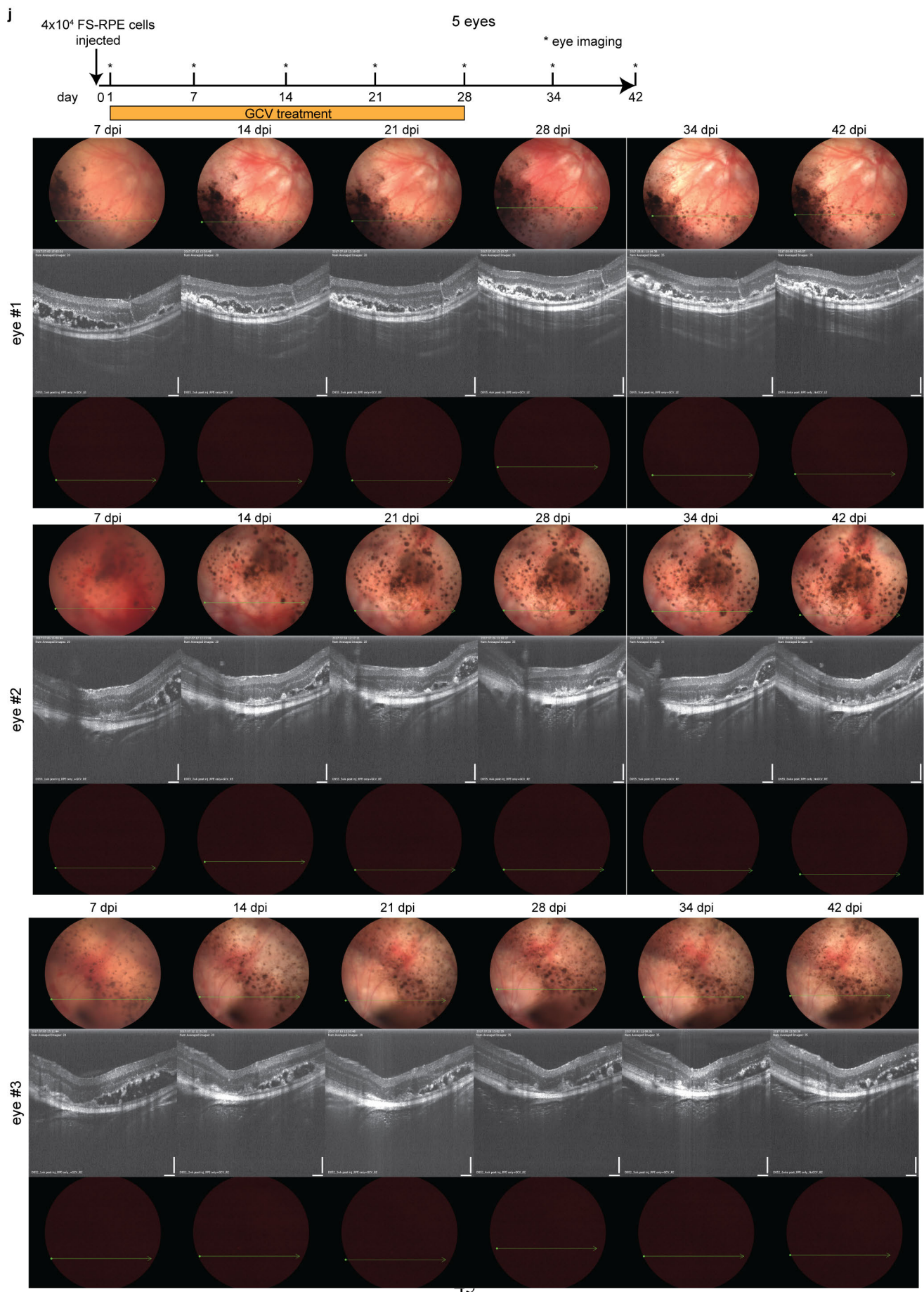
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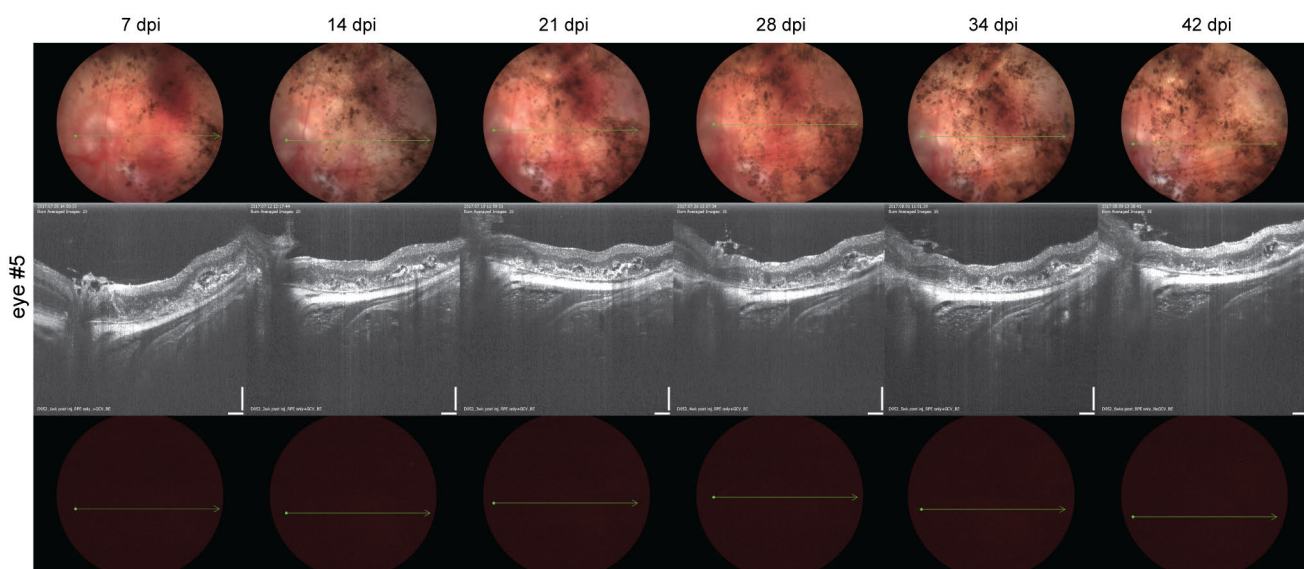
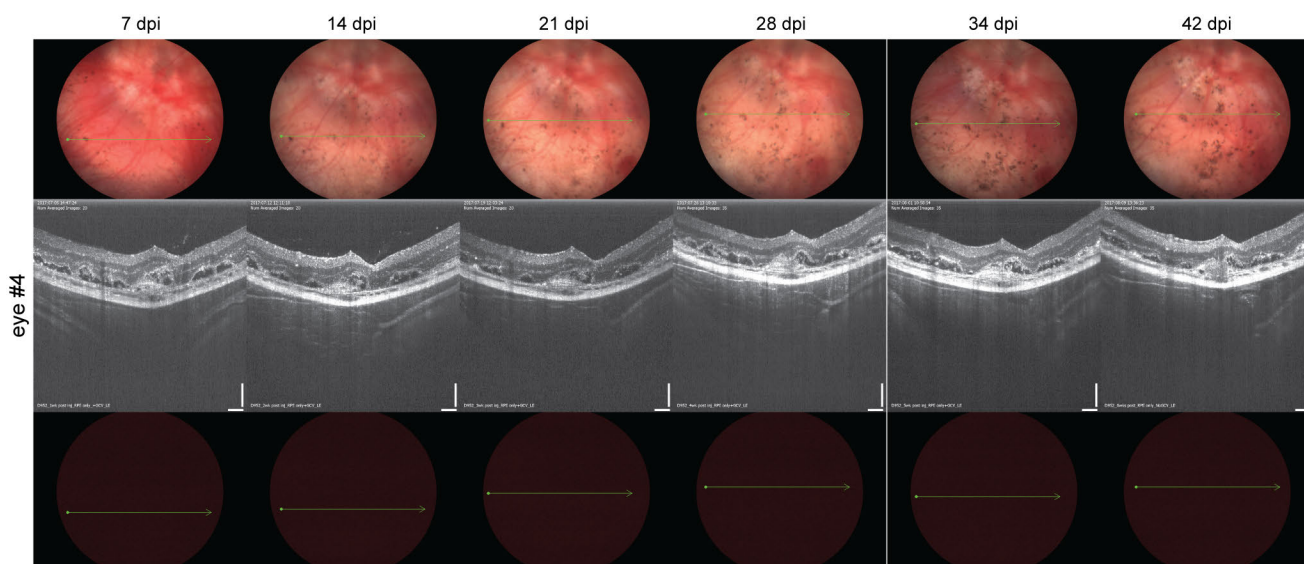




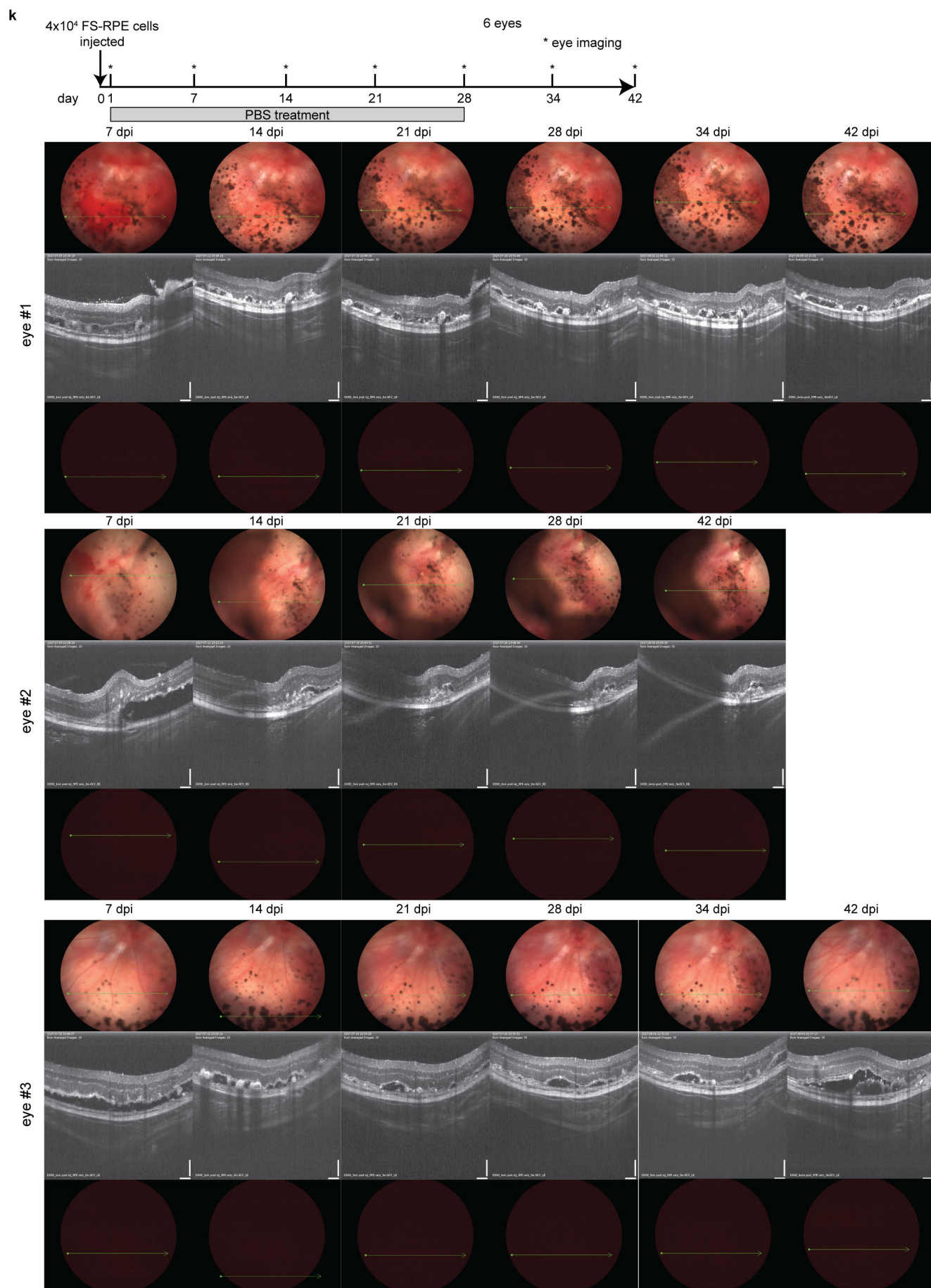
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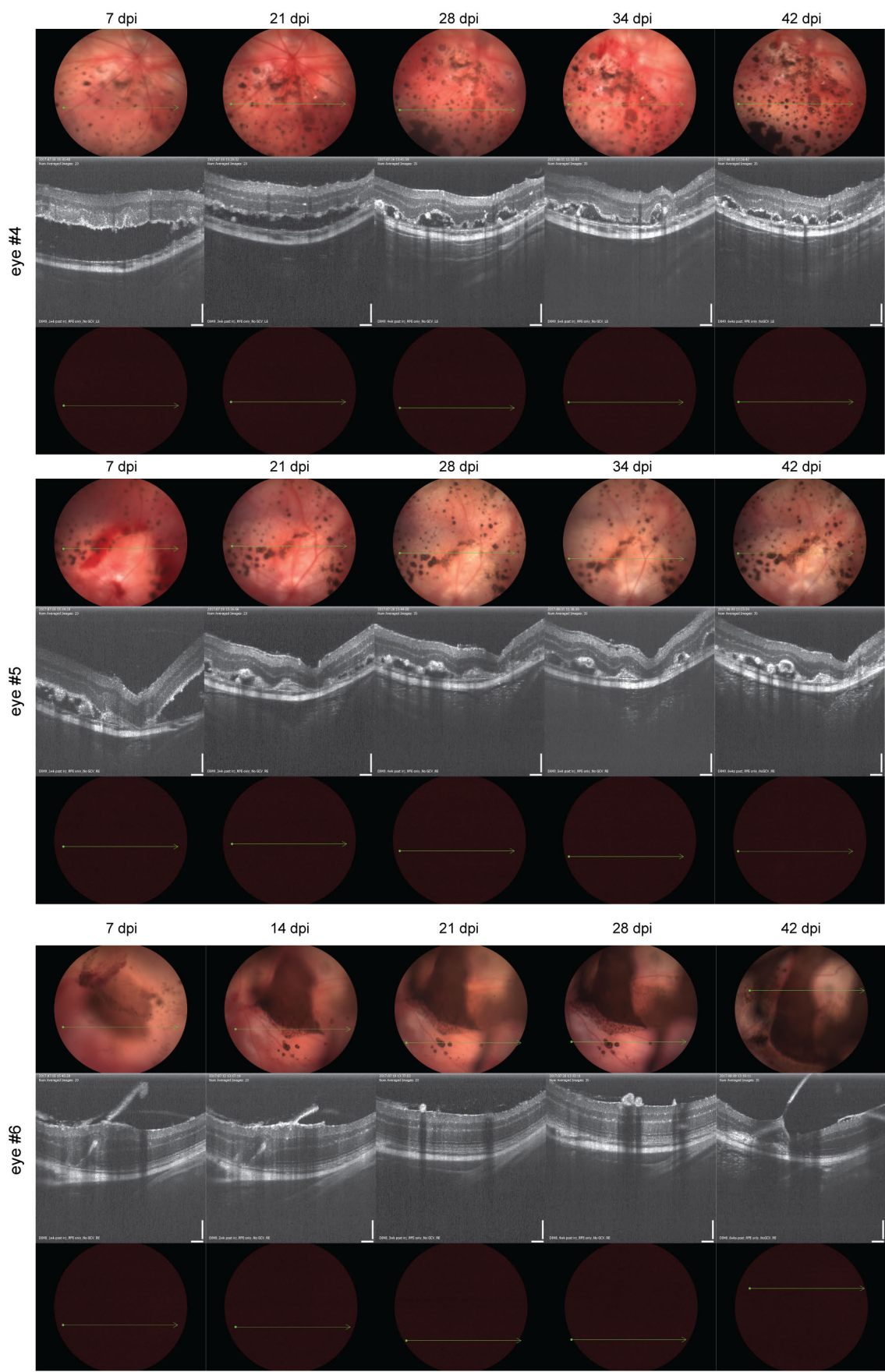


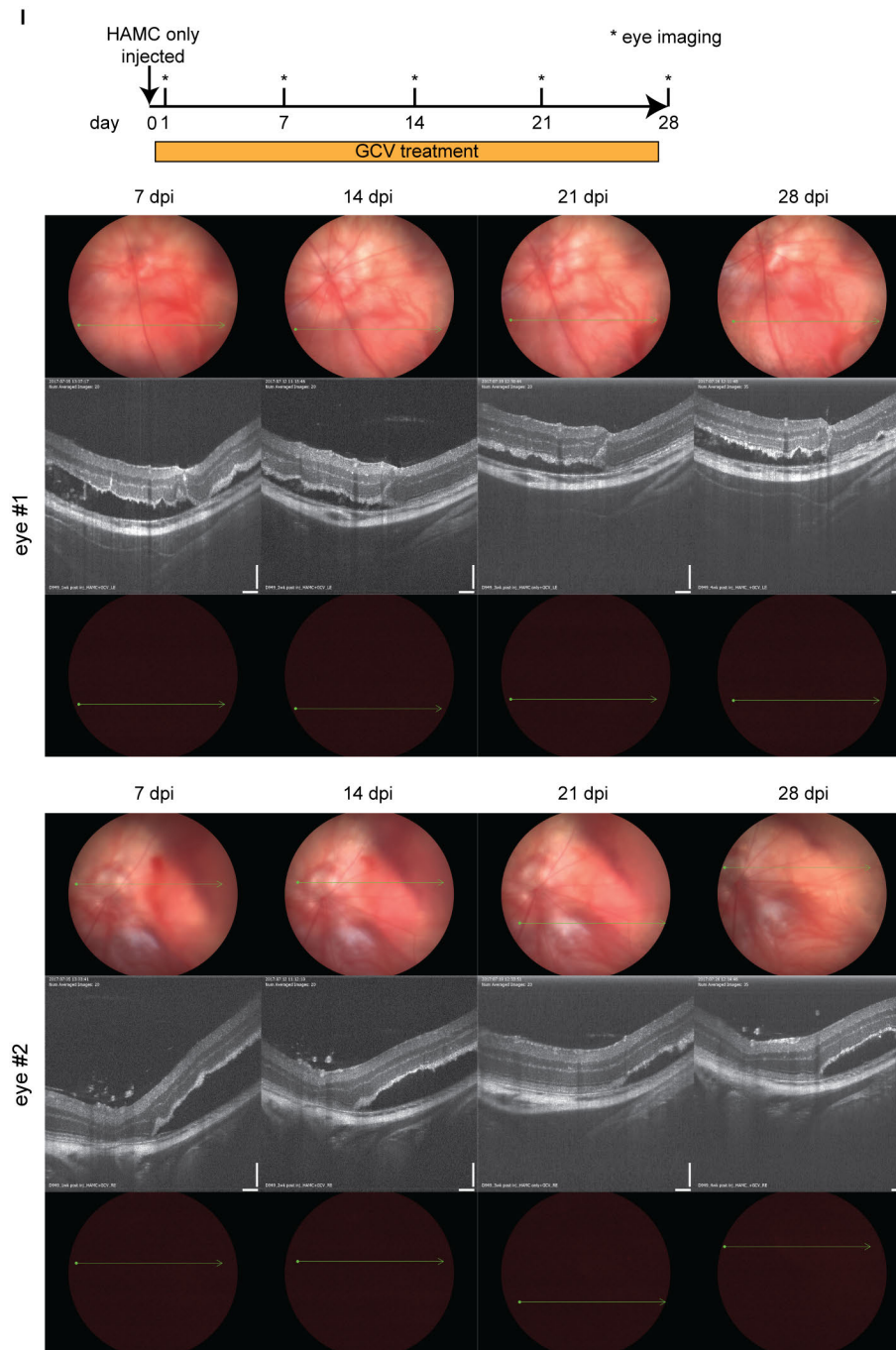




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Extended Data Figure 10 | Images of all eyes co-transplanted with FS-RPE and FS-ES cells (in addition to Fig. 4), and images of all the eyes transplanted only with FS-RPE cells. a-c, Fundoscopy, OCT and fluorescence imaging of eyes co-transplanted with FS-RPE and FS-ES cells (4-week GCV treatment). The absence of mCherry signal indicates that ES cell growth has not occurred. The bottom panel in **c** includes the green fluorescence channel to illustrate that the observed signal in the red fluorescence channel is actually autofluorescence. **d**, Histological analysis of the eye presented in **e**. **e-i**, Fundoscopy, OCT and fluorescence imaging of eyes co-transplanted with FS-RPE and FS-ES cells (PBS treatment). mCherry signal is detectable and indicates ES cell growth. GCV treatment began 3 weeks post-injection following an initial PBS treatment. **j**, Fundoscopy, OCT and fluorescence imaging of eyes receiving only FS-RPE cells (4-week GCV treatment). This demonstrates that GCV treatment did not affect the RPE cells. **k**, Fundoscopy, OCT and fluorescence imaging of eyes receiving only FS-

837 RPE cells (4-week PBS treatment). I, Fundoscopy, OCT and fluorescence imaging of eyes receiving
838 only HAMC (4-week GCV treatment).

Supplementary Calculation 1

The Effect of Aliquoting on FSL Calculation

We consider a pool of $N = 2^n$ cells that has $FSL_p = 1/p$ for some $p \in (0, 1)$. This means p is the probability that the pool of cells is not FS. Below we calculate first the probability that a randomly selected cell aliquot of size $M = 2^m$, $m < n$ is FS, i.e., the probability that it contains only FS cells.

In our model the pool can have 2^k non-FS cells with probability p_k , for $k = 0, 1, \dots, n$, such that $p_{k+1} = p_k/2$ for $k = 0, 1, 2, 3, \dots, n-1$. Thus

$$p_k = p_{k-1}/2 = p_{k-2}/2^2 = \dots = p_0/2^k,$$

for $k = 1, 2, \dots, n$, and since $1 - p$ is the probability that the pool is FS, we have

$$1 = 1 - p + \sum_{j=0}^n p_j = 1 - p + p_0(1 + 2^{-1} + \dots + 2^{-n}) = 1 - p + p_0(2 - 2^{-n}).$$

Hence

$$p_0 = \frac{p}{2 - 2^{-n}}, \quad p_k = p2^{-k}(2 - 2^{-n})^{-1}, \quad k = 0, 1, \dots, n.$$

Let q_a denote the probability that the aliquot is FS, and let $q_{a|k}$ denote the conditional probability that the aliquot is FS given that the pool has 2^k non-FS cells. Clearly, the conditional probability that the aliquot is FS, given that the pool is FS, is 1, and $q_{a|k} = 0$ if $N - 2^k < M$, i.e., when $k \geq n$. Thus by the law of total probability

$$q_a = 1 - p + \sum_{k=0}^n p_k q_{a|k} = 1 - p + p \sum_{k=0}^{n-1} 2^{-k}(2 - 2^{-n})^{-1} q_{a|k}. \quad (1)$$

It remains to calculate $q_{a|k}$. To this end we imagine that we choose M cells randomly, consecutively and without replacement, from a pool of size N which has $K := 2^k$ non-FS cells. The probabilities that the first chosen cell is FS, the second chosen cell is FS and that the j -th chosen cell is FS, are $\frac{N-K}{N}$, $\frac{N-K-1}{N-1}$ and $\frac{N-K-j+1}{N-j+1}$, respectively, for $j < N - K$. Hence

$$q_{a|k} = \frac{N-K}{N} \times \frac{N-K-1}{N-1} \times \dots \times \frac{N-K-M+1}{N-M+1} = \prod_{j=0}^{M-1} \frac{N-K-j}{N-j}. \quad (2)$$

Note that this value is the same as $\binom{N-K}{M}$ divided by $\binom{N}{M}$, which is immediately understandable by viewing the selection as randomly choosing M cells out of N . The number of all, equally probable selections is $\binom{N}{M}$, and among them $\binom{N-K}{M}$ are FS aliquots.

Now we calculate FSL_{ap} , the FSL of the randomly chosen aliquot. Using that $FSL_{ap} = 1/(1 - q_a)$, $FSL_p = 1/p$ and $1 - p = (FSL_p - 1)/FSL_p$, from equation (1) we get

$$FSL_{ap} = \frac{FSL_p}{1 - \sum_{k=0}^{n-1} 2^{-k}(2 - 2^{-n})^{-1} q_{a|k}}. \quad (3)$$

Notice that the contribution of the terms $2^{-k}(2 - 2^{-n})^{-1} q_{a|k}$ in the summation of the above expression is negligible for large k . Therefore we get a good approximation for FSL_{ap} if we keep only the terms with $k < m$. In this case we can simplify expression (2) for $q_{a|k}$ by

$$(N - K) \times (N - K - 1) \times \dots \times (N - M + 1)$$

to get

$$q_{a|k} = \frac{N-M}{N} \times \frac{N-M-1}{N-1} \times \frac{N-M-2}{N-2} \times \dots \times \frac{N-M-K+1}{N-K+1} = \prod_{j=0}^{K-1} \frac{N-M-j}{N-j}. \quad (4)$$

It is clear that

$$\frac{N-M-j}{N-j} < \frac{N-M}{N} = \frac{A-1}{A},$$

where $A = N/M$ is the number of aliquots generated from the pool of cells. Thus for $K := 2^k < M = 2^m$ we can approximate $q_{a|k}$ from above by

$$\left(\frac{A-1}{A}\right)^{2^k},$$

and from (3) we get the approximation

$$FSL_{ap} \approx \frac{FSL_p}{1 - \sum_{k=0}^{m-1} 2^{-k-1} \left(\frac{A-1}{A}\right)^{2^k}}. \quad (5)$$

We note that this approximation is very good in practice for the range of M and N that we are using, when M is essentially smaller than N , which is more than a million.

Finally we calculate the ratio FSL_{ap}/FSL_a , called FSL drop, where FSL_a denotes the FSL of a separately produced pool with the same cell number M as that of the aliquot. By our observations, in the clinically relevant range, the logarithm of the FSL is approximately a linear function of the logarithm of the cell number of the pool, with slope close to minus one. This means, that in the clinically relevant range of cell numbers we have

$$\frac{FSL_p}{FSL_a} \approx \frac{M}{N} = \frac{1}{A}.$$

Consequently, from (5) we obtain

$$\frac{FSL_{ap}}{FSL_a} \approx \frac{1}{A(1 - \sum_{k=0}^{m-1} 2^{-k-1} \left(\frac{A-1}{A}\right)^{2^k})}. \quad (6)$$

The contribution of those terms of the sum in this formula, which correspond to large k , is very small. In other words, for large M only the first few terms (i.e. 15) of the sum should be considered, since the contribution of the other terms is negligible. This means, that practically the FSL drop depends only on the number of aliquots A , when the cell numbers of the aliquot and of the pool are sufficiently large. To make this argument more precise let d_m denote the right-hand side of (6) and estimate the error $\varepsilon_l = d_l - d_m$ that we make when we sum the terms until $l-1 < m-1$ in place of $m-1$ in (6). Clearly, for any $l < m$ we have

$$\varepsilon_l = \frac{1}{A}((1 - S_m)^{-1} - (1 - S_l)^{-1}) \leq \frac{S_m - S_l}{A(1 - S_m)^2} < \frac{t_l}{A(1 - S)^2}, \quad (7)$$

where for integers $j \geq 0$ we use the notations

$$S_j := \sum_{k=0}^j 2^{-k-1} \left(\frac{A-1}{A}\right)^{2^k}, \quad S := \sum_{k=0}^{\infty} 2^{-k-1} \left(\frac{A-1}{A}\right)^{2^k}, \quad t_j := \sum_{k=j+1}^{\infty} 2^{-k-1} \left(\frac{A-1}{A}\right)^{2^k}.$$

Since $f(x) = 2^{-x}r^{-2^x}$ is a decreasing function of $x \in [0, \infty)$ for any $r \geq 1$, we get

$$t_l \leq \frac{1}{2} \int_l^{\infty} 2^{-x} r^{-2^x} dx = \frac{1}{2 \ln 2} \int_{2^l}^{\infty} y^{-2} r^{-y} dy \leq \frac{2^{-2l}}{2 \ln 2} \int_{2^l}^{\infty} r^{-y} dy = \frac{2^{-2l-1}}{\ln r \ln 2} r^{-2^l}, \quad (8)$$

with $r := A/(A-1)$. Notice that for every $j \geq 0$

$$1 - S \geq 1 - \sum_{k=0}^j 2^{-k-1} - t_j = 2^{-j-1} - 2^{-2j-1} (\ln r \ln 2)^{-1} r^{-2^j} = 2^{-j-1} (1 - 2^{-j} (\ln r \ln 2)^{-1} r^{-2^j}).$$

Thus choosing the smallest integer $j = j_0$ such that $j_0 \geq 1 - \log_2(\ln r \ln 2)$, we get

$$1 - S \geq 2^{-j_0-2} \geq 2^{-3} (\ln r \ln 2)$$

for all $A \geq 2$. Thus we have

$$(1 - S)^2 \geq 2^{-6} (\ln r \ln 2)^2.$$

Using this together with estimate (8), from (7) we obtain

$$\varepsilon_l < \frac{1}{A(\ln 2 \ln r)^3} 2^{-2l+6} r^{-2^l}$$

for all $l \leq m$ and $A \geq 2$, where $r = A/(A-1)$. Hence by the simple estimate

$$\ln r = \ln(1 + (A-1)^{-1}) \geq \ln 2(A-1)^{-1},$$

valid for all $A \geq 2$, and since $(\ln 2)^{-6} < 10$, we get

$$\varepsilon_l < 10 \frac{(A-1)^3}{A} 2^{-2l+6} r^{-2^l} < 10A^2 2^{-2l+6} r^{-2^l}.$$

Thus we can see that even for relatively large A , say $A = 512$, the error is negligible if $m > 15$ is replaced with 15 in the calculation of the FSL drop by the formula (6).